Role of Orphan Amino Acid Transporters and Interacting Proteins Studied in Mouse Models

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1. Summary

Amino acids play a critical role in the body as they constitute the building blocks of proteins and fulfill many other functions. The absorption of amino acids from the diet across small intestine enterocytes and the reabsorption of amino acids from the kidney glomerular filtrate are critical features that allow maintenance of the body's amino acid supply.

B^0AT1 (Slc6a19) is responsible for the uptake of a broad range of neutral amino acids across the apical membrane of kidney proximal tubule cells and small intestine enterocytes.
In kidney, we have shown in a collaborative study that correct apical expression of B^0AT1 and of other SLC6 family members B^0AT3/XT2 (Slc6a18), XT3 (Slc6a20b) and XT3s1/SIT1 (Slc6a20a) is controlled by the associated protein Tmem27/Collectrin (Danilczyk et al, 2006).
In the small intestine, we have shown in the present work that it is not Tmem27 but a homologue, the angiotensin-converting enzyme 2 (ACE2) that associates to B^0AT1. This association was demonstrated in *ace2* null mice as they lack B^0AT1 expression at the apical membrane of enterocytes. Furthermore, direct interaction between these two proteins was shown by co-immunoprecipitating ACE2 from small intestine brush border membranes with an antibody against B^0AT1. Study of the phenotype of *ace2* null mice evidenced a specific decrease in plasma glycine and L-tryptophan levels. Surprisingly, this was accompanied by an increase of L-lysine levels. Although the food intake of *ace2* null mice is similar to that of their wild-type littermates, they display a lower body weight at 8 weeks that originates from a slower weight gain after weaning.
Mutations in B^0AT1 have been shown to be causative of most cases of Hartnup disorder. We have shown that some of these mutations differentially interact with the associated proteins Tmem27 and ACE2, and postulate that this participates to the phenotypic heterogeneity of Hartnup disorder.
Altogether, our results show that B^0AT1 expression is controlled by tissue-specific associated proteins Tmem27 in kidney and ACE2 in small intestine. Interestingly, ACE2 has in contrast to Tmem27, a catalytic domain and plays a key role in the regulation of angiotensin II levels. This raises exciting questions concerning the regulation of amino acid transport by the renin angiotensin system that is involved in blood pressure regulation.

B^0AT3/XT2 (Slc6a18) has remained an "orphan transporter" since its identification in 1998. It localises to the later portion of the kidney proximal tubule in a complementary manner to the related B^0AT1 transporter (52% identity) where as B^0AT1 it associates to Tmem27 for correct apical localisation. The function of B^0AT3 as an amino acid transporter was only hinted after generation of the knock-out mouse model that displayed glycinuria (Quan et al, 2004).

However, direct evidence of amino acid transport was not generated as no substrate uptake was measured in *X. laevis* oocytes or other expression systems.

Co-expressing B^0AT3 with ACE2 in *X. laevis* oocytes, but intriguingly not with Tmem27, we succeeded in measuring a broad range amino acid transport (A, M, V, I > G, S, L). Further characterisation showed that L-isoleucine is transported with a relatively high affinity ($K_{0.5}$ = 0.21 mM compared to $K_{0.5}$ = 0.71 mM for B^0AT1) in a Na^+ and Cl^- -dependant and pH-independent manner. The organisation and the kinetics of the neutral amino acid transporters along the proximal tubule are therefore fitting to the decreased neutral amino acid content of the lumen along the proximal tubule with the expression of the higher affinity B^0AT3 in the later segments of the proximal tubule.

As expected, B^0AT3 expression in proximal kidney tubule was normal in *ace2* null mice confirming that Tmem27, and not ACE2, is necessary for correct expression of B^0AT3 in kidney proximal tubule cells. To assess B^0AT3 function, we re-analysed the *Slc6a18* (B^0AT3) knock-out mouse model after more than 10 generations of backcrossing into the C57BL/6 strain. Next to the previously published glycinuria, we observed a urinary loss of several other amino acids, in particular of beta-branched and small neutral ones. Using telemetry measurements, we confirmed the previously described link of B^0AT3 absence with hypertension but only in physically restrained animals.

Taken together, the data indicate that formerly orphan transporter XT2 functions as a Na^+ and Cl^- -dependent neutral amino acid transporter that we renamed B^0AT3.

2. Zusammenfassung

Aminosäuren spielen eine wichtige Rolle im Körper, da sie die Bausteine der Proteine darstellen und zusätzlich viele andere Funktionen erfüllen. Die Absorption von Aminosäuren aus der Nahrung durch die Enterozyten des Dünndarms und die Reabsorption der Aminosäuren aus dem glomerulären Filtrat der Nieren, sind kritische Funktionen, die die Erhaltung der Versorgung des Körpers mit Aminosäuren erlauben.

B^0AT1 (Slc6a19) ist für die Aufnahme einer Vielfalt von neutralen Aminosäuren über die apikale Membran von proximalen Tubuluszellen der Niere und der Enterozyten des Dünndarms verantwortlich. In einer kollaborativen Studie haben wir gezeigt, dass die korrekte apikale Expression von B^0AT1 und anderer SLC6 Familienmitglieder wie B^0AT3/XT2 (Slc6a18), XT3 (Slc6a20b) und XT3s1/SIT1 (Slc6a20a) in der Niere durch das assoziierte Protein Tmem27/Collectrin (Danilczyk et al, 2006) kontrolliert wird.
In dieser aktuellen Arbeit haben wir gezeigt, dass es im Dünndarm nicht Tmem27 ist, sondern ein Homolog, das Angiotensin-Converting Enzyme 2 (ACE2), welches mit B^0AT1 assoziiert. Diese Assoziation wurde in *ace2* null Mäusen gezeigt, da sie in der apikalen Membran der Enterozyten keine B^0AT1 Expression haben. Des Weiteren wurde die direkte Interaktion der beiden Proteine durch Co-Immunopräzipitation von ACE2 aus Bürstensaummebranen des Dünndarms mittels eines Antikörpers gegen B^0AT1 gezeigt.
Untersuchungen des Phänotyps von *ace2* null Mäusen erwiesen eine spezifische Senkung der Plasmakonzentrationen von Glycin und L-Tryptophan. Erstaunlicherweise ging dies mit einem Anstieg der L-Lysin-Werte einher. Obwohl die Nahrungsaufnahme der *ace2* null Mäuse ähnlich ist, wie die der Wildtyp Wurfgeschwister, weisen sie nach 8 Wochen ein kleineres Körpergewicht auf, welches auf eine langsamere Gewichtszunahme zurückzuführen ist.
Mutationen in B^0AT1 haben sich als die Ursache der meisten Fälle der Hartnup Krankheit erwiesen. Wir haben gezeigt, dass einige dieser Mutationen, auf verschiedene Weise, mit den assoziierten Proteinen Tmem27 und ACE2 interagieren, und postulieren, dass dies zur phänotypischen Heterogenität der Hartnup Krankheit beiträgt.
Insgesamt zeigen unsere Resultate, dass die Expression von B^0AT1 durch gewebespezifische assoziierte Proteine reguliert wird, Tmem27 in der Niere und ACE2 im Dünndarm. Interessanterweise hat ACE2 im Unterschied zu Tmem27 eine katalytische Domäne und spielt eine Schlüsselrolle in der Regulation der Angiotensin II Werte. Dies führt zu weiteren spannenden Fragen betreffend der Regulation von Aminosäuretransport durch das Renin-Angiotensin-System, welches in die Regulation des Blutdruckes involviert ist.

B^0AT3/XT2 (Slc6a18) ist ein so genannter “orphan transporter” geblieben seit seiner Entdeckung 1998. Dieser Transporter ist in den späteren Teilen des proximalen Tubulus der Niere zu finden, seine Lokalisation ist komplementär zu derer des verwandten B^0AT1 Transporters (52% Identität). Sowohl B^0AT3 als auch B^0AT1 werden mit Tmem27 assoziiert vorgefunden, welches sich als notwendig für eine korrekte apikale Lokalisation herausgestellt hat. Ein möglicher Aminosäuretransport durch B^0AT3 wurde nur durch Verwendung des knockout-Maus-Modells angedeutet, welches Glycinuria zeigte (Quan et al, 2004). Ein direkter Beweis war allerdings nicht möglich, da in *X. laevis* Ooozyten oder anderen Expressionssystemen keine Aufnahme von Substrat messbar war.

Durch die Co-Expression von B^0AT3 mit ACE2 in *X. laevis* Oozyten, aber nicht mit Tmem27, waren wir in der Lage eine grosse Vielfalt von Aminosäurentransport zu messen (A, M, V, I > G, S, L). Weitere Charakterisierung zeigte dass L-Isoleucin mit einer relativ hohen Affinität ($K_{0.5}$ = 0.21 mM verglichen mit $K_{0.5}$ = 0.71 mM für B^0AT1) auf eine Na^+ und Cl^- -abhängige und pH-unabhängige Art transportiert wird. Die Organisation und die Kinetik des Transports neutraler Aminosäuren entlang des proximalen Tubulus passen daher zum erniedrigten Gehalt an neutralen Aminosäuren im Lumen entlang des proximalen Tubulus, wobei der höher affine B^0AT3 Transporter in den späteren Segmenten des proximalen Tubulus exprimiert ist.

Wie erwartet war die B^0AT3 Expression im proximalen Nierentubulus in den *ace2* null Mäusen normal, dies bestätigt, dass Tmem27 und nicht ACE2 notwendig sind, für die korrekte Expression von B^0AT3 in den proximalen Nierentubuluszellen. Um die Funktion von B^0AT3 zu bestimmen haben wir nochmals das *Slc6a18* (B^0AT3) knockout-Maus-Modell nach mehr als 10 Rückkreuzungsgenerationen mit dem C57BL/6 Stamm analysiert.

Neben der bereits zuvor publizierten Glycinuria, haben wir den Verlust verschiedener anderer Aminosäuren in den Urin beobachtet, insbesondere von beta-verzweigten und kleinen neutralen Aminosäuren. Unter Verwendung von Telemetrie-Messungen haben wir den zuvor beschriebenen Zusammenhang zwischen der Abwesenheit von B^0AT3 mit Bluthochdruck bestätigt, dies jedoch nur in physisch eingeschränkten Tieren.

Zusammenfassend zeigen die Daten, dass der ehemals als „orphan transporter“ beschriebene XT2 als Na^+ und Cl^- -abhängiger neutraler Aminosäurentransporter funktioniert, welchen wir aufgrund dieser Eigenschaften in B^0AT3 umbenannt haben.

3. General Considerations on Nutrition

3.1 Metabolism and Energy

The body's metabolism is the set of chemical processes involved in energy production, energy release and growth that are necessary to maintain life. The first law of thermodynamics states that total energy in a closed system is constant. The energy acquired by the body is used to fuel a variety of processes described in figure 1, accounting for a basal metabolic rate of about 30 kcal / kg body weight / day. Additionally, the second law of thermodynamics states that chemical transformations lead to loss of free energy. The body therefore continuously requires new sources of energy, which in humans is mostly acquired from ingested food.

The western diet consists of 55-60% carbohydrates, 25-30% fat and 10-15% proteins. Each of these compounds can be digested to smaller molecules that are absorbed in the small intestine. The fate of sugars, lipids and amino acids will depend on the body's energy requirements. These compounds can either be used for the synthesis of new molecules, converted to energy or stored. Carbohdydrates and lipids can be glycolysed (in muscle and liver) and beta-oxidized (in liver), respectively, before integration in the citric acid cycle and oxidative phosphorylation. These metabolic pathways lead to the synthesis of adenosine triphosphate (ATP), that contains high energy in one of its phosphate bonds. The integration of amino acids in energy metabolism will be discussed further in section 3.3. Carbohydrates can be stored as glycogen in muscle or liver, triglycerides in the adipocytes of fat tissue (and partly in muscle) and amino acids as proteins in muscle (Boron & Boulpaep, 2005).

3.2 General Considerations on Proteins

Proteins are essential for nutrition because of the constituent amino acids that make up all the synthesised proteins of the body. Though it was generally believed that animal proteins were exclusively constituted of L-amino acids, D-amino acids are also physiologically relevant. D-serine acts as a neurotransmitter in the brain (Wolosker et al, 2008), and D- to L-aspartic acid ratio increases in elderly tissue, modifying protein structure (Fujii, 2005). This remains the exception and amino acids mentioned all through the text refer to L-amino acids, unless otherwise specified.

The western diet daily supplies about 0.8 g protein / kg body weight, which can be converted to 4.3 kcal / g of amino acids. Proteins account for 14% of the body mass, and are distributed

throughout the body with 40% in muscle, 25% in body organs, and the remaining mostly in skin and blood. From all the proteins contained in the body, half can be mobilised as a fuel source. However, since most proteins have roles in structure or function, only 5% of the resting energy comes from proteins, which may be increased up to 15% in starvation conditions (Boron & Boulpaep, 2005; Gropper et al, 2005).

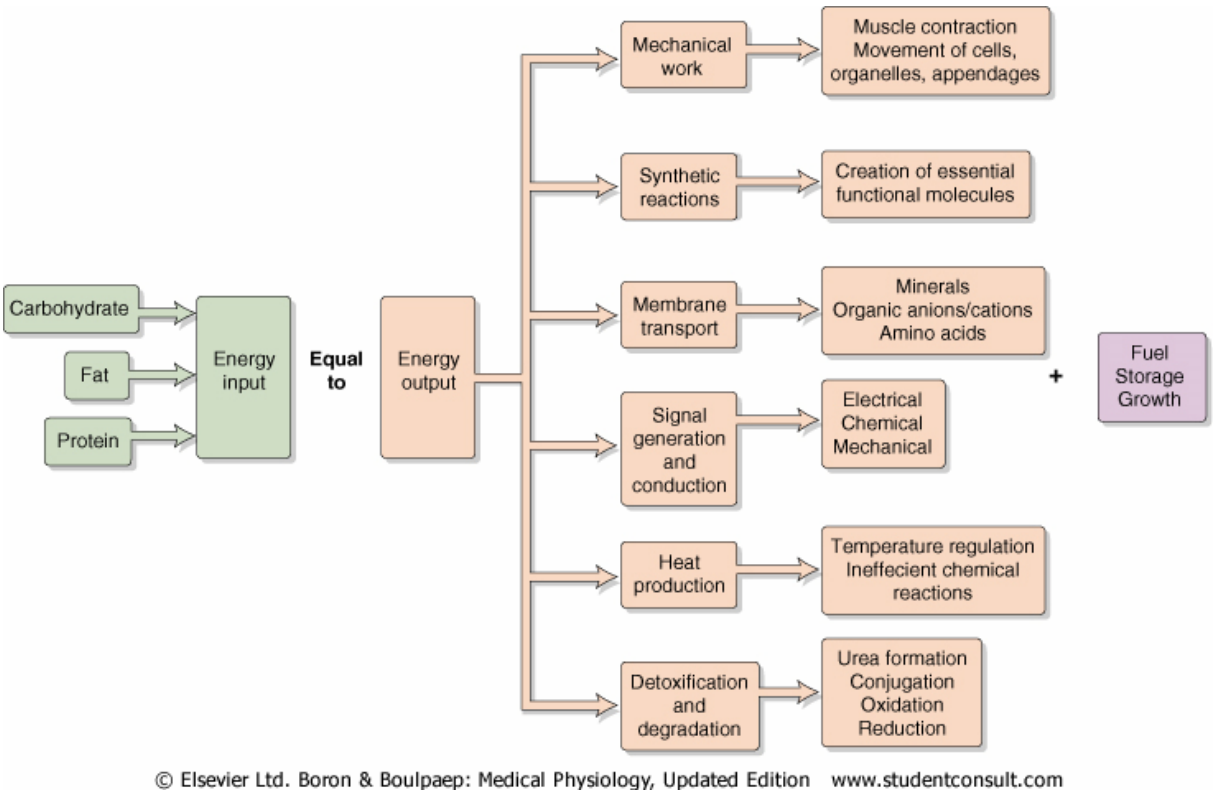

Figure 1. Energy balance (Boron & Boulpaep, 2005).

Protein functional categories

Enzymes: Catalyse chemical reactions and allow, among others, digestion, tissue energy production, blood coagulation, excitability and contractility of neuromuscular tissue.

Hormones (except steroid hormones)**:** Act as chemical messengers by binding to a receptor and generally regulate metabolic processes (insulin, glucagon). They are synthesised and secreted by endocrine tissues (glands). Amino acids are often precursors for hormone synthesis like tyrosine for catecholamine synthesis and tryptophan for melatonin synthesis.

Structural proteins: Contractile proteins (actin and myosin) or fibrous proteins present in bone, teeth, skin, tendons, cartilage, blood vessels, hair and skin (collagen, elastin, keratin).
Immunoproteins: Protect the organism against disease (immunoglobulins or antibodies).
Transport proteins: Transport specific substrates in blood (albumin), within cells, or across the membrane (amino acid transporters).
Other functions: Blood pH buffering or conjugation of proteins with non-protein components to form glycoproteins (mucus, connective tissue), proteoglycans (extracellular matrix, bone, cartilage), lipoproteins (chylomicrons, very low / low / high density lipoproteins), flavoproteins (redox potential) and metalloproteins (enzymatic or transport functions) (Gropper et al, 2005).

3.3 Amino Acid Metabolism

Once taken up by the small intestine as free amino acids or small peptides (see section 4.3), most amino acids are released in the portal circulation either intact or after conversions to other amino acids. Part of the absorbed amino acids remain in small intestine enterocytes to participate in local protein synthesis (house-keeping functions, apoproteins, digestive enzymes, hormones, nitrogen containing compounds). Glutamine for example is highly used for energy production in small intestine enterocytes. Amino acids released in the portal circulation are highly taken up by liver periportal hepatocytes (up to 50% after a meal) where they may be converted to other amino acids depending on the body's requirements and/or released back into the circulation. Exceptions are glutamate and aspartate that are mostly taken up by perivenous hepatocytes and branched chain amino acids (valine, isoleucine and leucine) that are mostly taken up by muscle. Amino acids present in the circulation after a meal are quickly absorbed by the tissues leaving only a small pool of amino acids behind that mixes with the body's protein degradation products ([total plasma amino acids] = 2.4 mM ≈ 1.5 g / 3 L plasma). Despite this small amount, amino acid reabsorption by the kidneys is critical and reaches 95-99%. Once amino acids reach their target destination in the cells, they will either participate in protein synthesis or nitrogen containing compounds synthesis like glutathione, carnitine, creatine, carnosine and choline.

Adaptation of the body's amino acid requirements to the diet's amino acid composition partly relies on the ability to synthesise new amino acids from existing substrates. A second crucial characteristic of a diet's nutritive impact is the presence of essential amino acids (typically 20%), i.e. amino acids that cannot be synthesised by the body (Table 1). The first step of amino acid conversion is either deamination or transamination. Deamination is the process of

removing the ammonia group from an amino acid either obtaining another amino acid or an α-keto acid (or carbon skeletton) using specialised enzymes (lyases, dehydratases, dehydrogenases). Transamination, on the other hand, refers to the transfer of the amino group of one amino acid to another one or to an α-keto acid through the action of transaminases. Glutamate and α-ketoglutarate readily donate or accept amino groups and thereby play an important role in amino acid metabolism. Amino acids that cannot be synthesised through the process of transamination are defined as essential, and have to be acquired through the diet. This classification is however flexible as some amino acids can be conditionally or acquired essential for infants and growing children, during organ failure, premature birth[†] or cirrhosis[§] (Table 1).

Essential	Non-essential
Leucine	Alanine
Isoleucine	Cysteine[§†°]
Valine	Glutamine
Histidine	Glycine
Threonine	Serine
Methionine	Threonine
Tryptophan	Asparagine
Phenylalanine	Aspartate
Lysine	Proline[†°]
	Tyrosine[§]
	Arginine°

Table 1. Essential and non-essential L-amino acids. Conditional essential amino acids during growth°, premature birth[†] or cirrhosis[§]. Modified from (Gropper et al, 2005).

The toxic ammonia freed during deamination of amino acids will be converted to urea by integration in the urea cycle. Urea can then be transported to the blood and is mostly eliminated in the urine. This is also the case for ammonia acquired from the diet, or from bacterial degradation of amino acids. Ammonia in the portal circulation is usually taken up by uragenic periportal hepatocytes, unless when present in excessive amounts leading to uptake by perivenous hepatocytes and integration in glutamine synthesis. The situation is similar in non-uragenic peripheral tissues where ammonia associates to glutamate to produce glutamine that is then transported to liver or kidney for catabolism.

There are multiple fate possibilities for the α-keto acid left over from de- or trans-amination depending on the amino acid it originates from and on the body's energetic state. All α-keto acids can be fully oxidized to provide energy, reaching up to 50% of the liver's energetic needs. Besides oxidation, α-keto acids may lead to the synthesis of glucose, ketone bodies, cholesterol or fatty acids.

Glucogenic amino acids can be catabolised to components of the citric acid cycle leading to glucose synthesis. This situation is favoured by high glucagon:insulin ratios or glucocorticoids (cortisol). Ketogenic amino acids are catabolised to acetyl-CoA or acetoacetate that can then be converted to ketone bodies (Table 2). This is favoured by diets insufficient in carbohydrates. Fatty acids may be synthesised from amino acids in case of energy excess and sufficient dietary protein and carbohydrate supply (Gropper et al, 2005).

Glucogenic	Yielded intermediate	Ketogenic	Yielded intermediate
Alanine **Glycine** **Serine** **Cysteine** Tryptophan Threonine*	Pyruvate	**Leucine** Tyrosine Phenylalanine	Acetoacetate
Glutamate **Histidine** **Arginine** **Proline** **Glutamine**	α-ketoglutarate	**Lysine** Isoleucine Leucine Tryptophan Threonine*	Acetyl-CoA
Valine **Methionine** Threonine Isoleucine	Succinyl-CoA		
Aspartate Phenylalanine Tyrosine	Fumarate		
Asparagine **Aspartate**	Oxaloacetate		

Table 2. Glucogenic and ketogenic amino acids with respective yielded intermediates during glucose and ketone bodies synthesis, respectively. Exclusively glucogenic or ketogenic amino acids are indicated in bold. * Physiological contribution unclear. Modified from (Gropper et al, 2005).

4. Amino Acid Transport across Epithelia

4.1 Epithelial Structure and Common Properties

While it is necessary for the body to acquire nutrients from external sources, it is also important to protect and regulate the inside milieu. Performing both tasks is possible by the presence of the epithelium, a sheet of cells that line all of the body's contact areas with the outside. The movement of solutes across this dynamic barrier is regulated and can follow two paths, the paracellular route (between the cells) and the transcellular route (through the cells).

The paracellular route is controlled by an essential feature of the epithelium, the tight junctions or *zona occludens*. These protein complexes allow close association of two adjacent cells thereby increasing their adherence and restraining free diffusion of solutes. Indeed, tightness of an epithelium is proportional to the amount of tight junctions. Another important function of the tight junctions is to set a boundary between the apical membrane usually facing the outside, and the basolateral membrane facing the extracellular fluid. Such a barrier allows polarization of the cells by keeping the proteins and lipids in the membrane they have been sorted to. This allows distinct but complementary membrane functions and vectorial transport against concentration and/or electrical gradients. For example, the Na/K-ATPase is almost always restricted to the basolateral membrane. Other junctional complexes include adhering junctions (*zona adherens*, allowing cell-to-cell contact), gap junctions (cytosol connection) and desmosomes (*macula adherens*, for structural stabilisation) (Figure 2). Structural support and help in architectural organisation is given by the basement membrane which is secreted by the basolateral membrane. It consists of extracellular matrix proteins (collagen, laminin, proteoglycans,…) and informs the cell on orientation.

Passage of polar molecules through the transcellular route is protected by the hydrophobic interior of the lipid bilayer in the cell membrane. Transcellular movement of water-soluble organic molecules therefore requires the presence of specific transport proteins. The expression of these transport proteins depends on the function of the organ delimited by the epithelium, but protein expression pattern is also heterogeneous within each organ, it can vary along the proximal-distal tubule axis in kidney as well as on the villus-crypt axis in the small intestine.

In epithelial cells, many transport proteins and enzymes localize to the membrane. For efficient transport, the apical membrane surface is often amplified (up to 20 times) by

microvillar projections that constitute the brush border. The basolateral membrane may also have lateral interdigitations and basal infoldings, though to a smaller extent than on the apical side (Boron & Boulpaep, 2005).

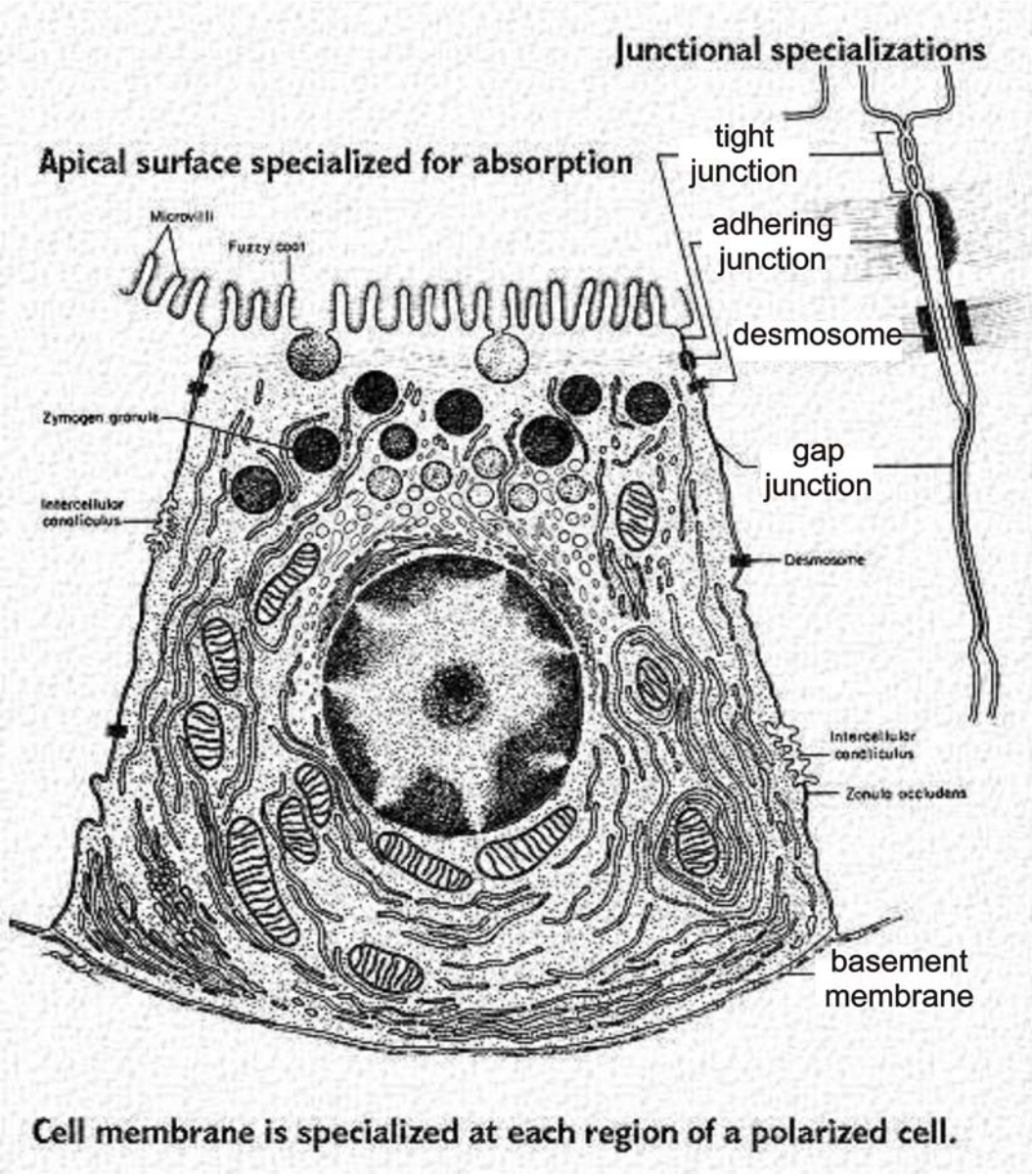

Figure 2. Epithelial cell scheme. Source : http://mednote.co.kr/images/membr-junction.jpg

4.2 Intestinal Absorption

4.2.1 Organisation of the Gastrointestinal Tract

The gastrointestinal tract is composed of a tube of hollow organs stretching from the mouth to the anus, complemented by organs adding secretions. The hollow organs are separated by sphincters, allowing specialisation and the sequential processing of food (Figure 3). Food is first chopped in small pieces and lubricated in the mouth and oropharynx, where carbohydrate and fat digestion begins. Ingested food is then propelled to the stomach through the oesophagus. Gastric content is then transferred to the small intestine where digestion continues and nutrient absorption primarily occurs. Fluid and electrolyte absorption and secretion take place in the small as well as in the large intestine which additionally serves as storage of fecal matter.

The wall of the gastrointestinal tract is organised in a mucosa, a submucosa, a muscle layer and a serosa. The mucosa is constituted of an epithelial cell layer and loose connective tissue (*lamina propria*), as well as capillaries, enteric neurons, immune cells and a thin layer of smooth muscle *(lamina muscularis mucosae*). The small intestine epithelial cell layer is organised in evaginated villi where the absorption of nutrients and electrolytes takes place, and invaginated crypts of Lieberkühn that are responsible for secretion (Figure 3). This structure is additionally organised in folds of Kerckring, increasing the absorptive surface to a total of 200 m^2 in the small intestine. The submucosa consists of loose connective tissue, larger blood vessels and glands. There are two muscle layers (*muscularis externa*) surrounding these structures, a circular and a longitudinal one which generate the peristaltic movement and receive the enteric neuron projections. The serosa, an envelope of connective tissue covered by squamous epithelial cells finally covers the whole tract.

The small intestine is a dynamic organ with proliferation of epithelial cells at the base of the crypt which then migrate towards the tip of the villus. The progenitor or stem cell differentiates to a vacuolated cell, a goblet cell, a Paneth cell or an enteroendocrine cell. Vacuolated cells then differentiate to villous absorptive cells that migrate to the tip of the villus and slough in the lumen over a 48-96 hours cycle, depending on the fasting/feeding state. Goblet cells secrete mucus, Paneth cells provide host defense against bacteria or bacterial antigens, and enteroendocrine cells sense the luminal environment and secrete hormones on the blood side. There the hormones modulate intestinal motility, augment pancreatic β-cell response or induce satiety (gastrin, cholecystokinin, glucose-like peptide 1, Peptide YY) (Cummings & Overduin, 2007).

Function of the gastrointestinal tract is regulated by the enteric nervous system (ENS) with sensory neurons, interneurons and secretomotorneurons (total of 100 million neurons). The ENS constitutes in itself a complete reflex circuit stimulating or inhibiting smooth muscle cells, epithelial cells, enteroendocrine cells and submucosal vessels. The ENS is additionally under the control of the autonomic nervous system, hormones as well as the immune system (Boron & Boulpaep, 2005).

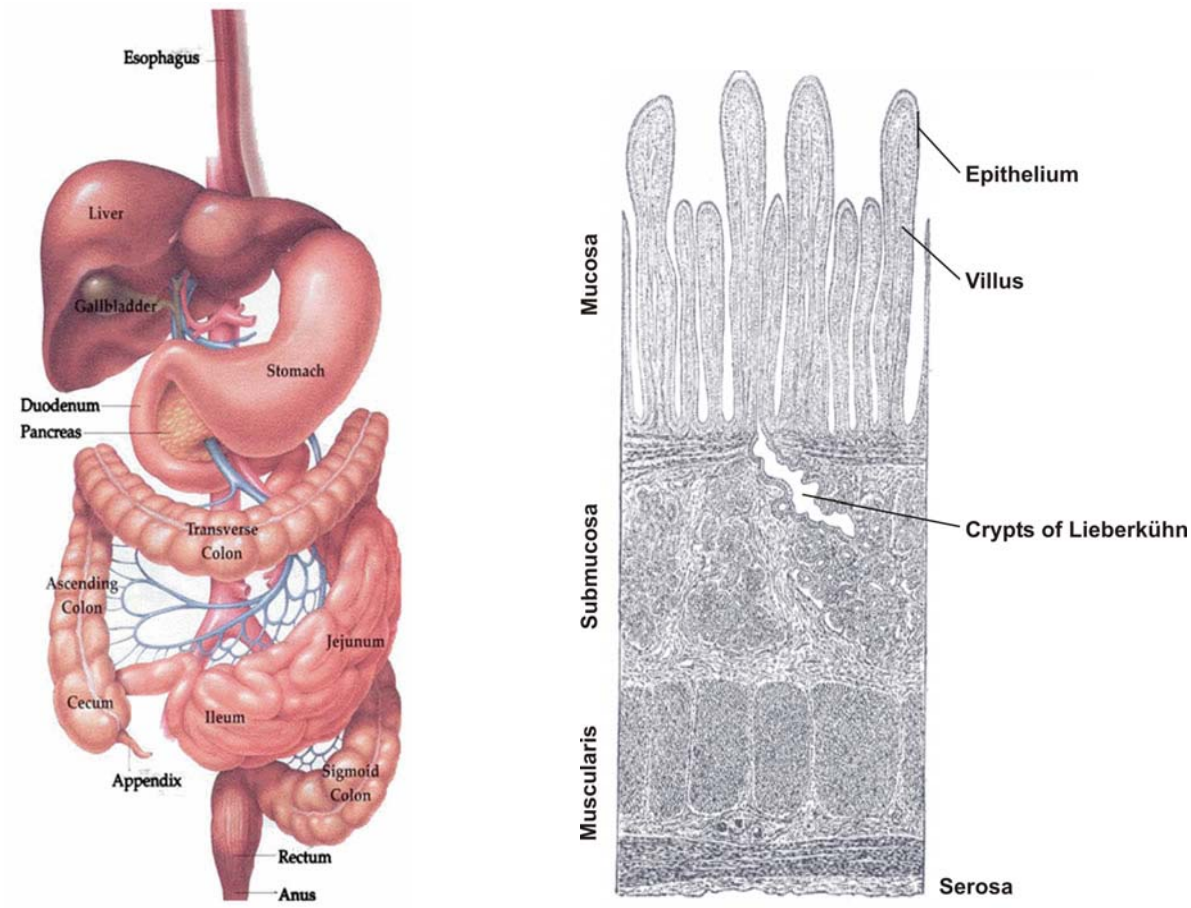

Figure 3. Gastrointestinal tract and intestinal section scheme (Gray, 1918).

4.2.2 Protein Digestion

Digestion is the process of converting complex dietary substances to absorbable molecules. It occurs through mechanical and chemical processing.

To be absorbed, proteins are enzymatically digested to free amino acids or di- and tri-peptides by gastric proteases in the stomach and by pancreatic enzymes and brush border dipeptidases in the small intestine. Inside the enterocytes, di- and tri-peptides are hydrolysed to single amino acids by cytosolic enzymes. Amino acids are then released in the portal circulation. Protein digestion and uptake of amino acids is a very effective process with less than 4% of ingested nitrogen going in stool. There is only an antigenic amount of protein that is absorbed intact in the small intestine, except in neonates where it plays an important role for dietary protein immune response.

There are 70 to 100 g of protein ingested every day, which is complemented by the digestion of endogenously secreted proteins. This means that half of the proteins entering the small intestine are enzymes, hormones, and immunoglobulins from salivary, gastric, pancreatic, biliary and jejunal secretions (17 g / day), as well as desquamated epithelial cells (50 g / day). Protein digestion is influenced by the amino acid composition (proline-rich is less digested), the source of the diet (vegetal less digested), and food processing (cooking, storage).

Chemical digestion of proteins starts in the stomach where HCl is secreted by the gastric parietal cells in response to various stimulations (gastrin, acetylcholine, histamine), and will denature quaternary, tertiary and secondary structures without affecting peptide bonds. Gastric and pancreatic enzymes are usually converted from a proenzyme to an active enzyme form to avoid auto-digestion processes. The proenzyme pepsinogen secreted by gastric chief cells is converted to pepsin by HCl, as well as by the newly formed pepsin (positive feedback mechanism). Pepsin is a low specificity endopeptidase digesting 10-15% of proteins with high activity at pH < 3.5, generating large polypeptides, oligopeptide and free amino acids. The stomach content is then delivered to the small intestine through the pylorus where it will stimulate the secretion of regulatory hormones/peptides such as secretins like cholecystokinine (CCK) from mucosal enteroendocrine cells into the blood (Cummings, Overduin). These secretins reach the pancreas and acinar cells which then secrete alkaline pancreatic juice, electrolytes, water and digestive proenzymes/zymogens.

Intestinal brush border enzymes convert the proenzyme trypsinogen to trypsin, that in turn converts chymotrysinogen to chymotrypsin, and the procarboxypeptidases to carboxypeptidases. Brush border peptidases cleave 3-8 amino acids oligopeptides, acting as endo- or exo-peptidases from the C or the N terminus (Table 3) (Boron & Boulpaep, 2005;

Gropper et al, 2005). The relatively fewer cytosolic peptidases cleave specific bonds in 2-3 amino acids peptides.

Amino acids are finally taken up by the enterocytes either in the form of di- and tri-peptides by the proton coupled peptide transporter (PepT1, Slc15a1), or in the form of free amino acids by amino acid transporters (see section 5).

Proenzyme production site	Enzyme	Activity site	Substrate (peptide bonds adjacent to)
Stomach	Pepsin	Endopeptidase	Most AA[1]
Pancreas	Trypsin Chymotrypsin Elastase	Endopeptidases	Basic AA Aromatic AA
	Carboxypeptidase A	Exopeptidases	C_{ter}[2] Neutral AA
	Carboxypeptidase B		C_{ter} Basic AA
Brush border	Aminopeptidases Dipeptidylpeptidase Tripeptidylpeptidase	Exopeptidases	N_{ter}[3]

Table 3. Overview and mode of action of some digestive enzymes. [1]AA: amino acids. [2]C_{ter}: carboxy terminal. [3]N_{ter}: amino terminal. Modified from (Gropper et al, 2005).

4.3 Kidney Reabsorption

4.3.1 Anatomy and Physiology of the Kidney

The kidney is a paired, bean-shaped organ of 115-170 g in human that filters toxins and metabolic products, assures homeostasis of acids and bases, electrolytes and water and also produces hormones for erythrogenesis, calcium metabolism, regulation of blood pressure and blood flow.

An opening called the hilus is the entry point for the renal artery and nerves and the exit point for the renal vein and the lymphatic system. Macroscopic kidney structure, from inside to

outside, is arranged in renal sinus, medulla and cortex. The renal sinus collects urine formed from the glomerular filtrate by the nephron, directing it to the bladder for storage. In the medulla, tubules are arranged in parallel and organised in 8-18 conically shaped pyramids, whereas in the cortex the tubules are organised in convoluted structures (Figure 4).

The functional unit of the kidney is the nephron (800'000 to 1'200'000 in human and 100'000 in mouse) which consists of the glomerulus and the tubule. The glomerulus is a cluster of blood vessels that form the filtrate. The tubule is an epithelial structure that through the action of specialised subdivisions forms urine from the glomerular filtrate. These two structures join at Bowman's capsule, which is contiguous to the tubule lumen.
Nephrons are organised in two categories depending on glomerulus localisation and extension of the loop of Henle. Glomeruli of superficial nephrons are in the cortex and the loop of Henle only extends till the outer and inner medulla, while juxtamedullary nephrons have their glomeruli at the limit between cortex and medulla, with the loop of Henle reaching until the tip of the medulla.

The renal corpuscule is where blood is filtered from the capillary network to the Bowman space in the Bowman capsule. The glomerular filtration barrier consists of endothelial cells forming fenestrated capillaries surrounded by the glomerular basement membrane and epithelial podocytes, with the exception of the center where endothelial cells are in direct contact with the mesengial cells.
A first filtration occurs through the 70 nm fenestrations that only restrict cellular elements. The basement membrane consists of 3 layers and restricts the passage of medium to large solutes (> 1 kDa) and additionally possesses a strong anionic charge. The podocyte foot processes are separated by filtration slits that are covered by a thin structure, the slit diaphragm, with 4 to 14 nm diameter pores. A network of contractile mesangial cells in the capsule supports the structure by secreting an extracellular matrix.

Even though kidneys only represent about 0.5% of the total body weight, they receive up to 20% of the cardiac output. The vascular elements of the renal circulation are a high-resistance afferent arteriole, a high-pressure glomerular capillary network, a high-resistance efferent arteriole and a low pressure capillary network that surrounds the tubules and takes up the reabsorbed fluid.
Pressure in the afferent and efferent arterioles determine the hydrostatic pressure in the glomerular capillaries. The arteriole tone is controlled by sympathetic innervation and chemical mediators, and there is no parasympathetic innervation in the kidney. Blood flow in the cortex comes from efferent arterioles of superficial glomeruli, receiving 90% of renal

artery blood, while blood flow in the medulla originates from juxtamedullary glomeruli efferent arterioles that form the vasa recta and receives the remaining 10% blood supply. Lymph vessels drain interstitial fluid of the cortex and contain high levels of hormones (e.g. renin), but not the one of the medulla which would otherwise wash the osmotic gradient away.

The juxtaglomerular apparatus (JGA) is an important structure that regulates renal blood flow, filtration rate, Na^+ balance and systemic blood pressure. It is composed of an extraglomerular matrix, the macula densa cells and the granular cells. The macula densa cells are specialised cells of the thick ascending limb that contact the glomerulus. The granular cells, or juxtaglomerular cells, are specialized smooth muscle cells from the afferent arteriole that produce, store and release renin (see section 6.1). The JGA also allows tubuloglomerular feedback which induces the decrease of glomerular filtration rate upon increases of fluid or NaCl concentration at the macula densa.

Tubule function relies on its highly specialised segments that contain specialised transporters. The proximal tubule is divided in convoluted segments S1 and S2 (PCT: proximal convoluted tubule) and the straight segment S3 (PST: proximal straight tubule). The richly folded brush border membrane of this segment allows high reabsorption rates of nutrients (amino acids, glucose), NaCl, $NaHCO_3$, water, and divalent ions ($PO4_2^-$, Ca_2^+, $SO4_2^-$), as well as secretion of NH_4^+ and many other solutes.
This segment is followed by the loop of Henle that ends at the macula densa. Its role is to concentrate or dilute urine. For this purpose, a hyperosmotic interstitium gradient is generated in the medulla by the juxtamedullary nephrons. This gradient may or may not be used to concentrate urine in the distal medullary collecting ducts depending on the body's requirements.
The distal tubule and the collecting duct system are responsible for the fine control of water and salt excretion. The distal convoluted tubule (DCT) starts at the macula densa and is followed by the connecting tubule (CNT) and the cortical collecting ducts (CCD). CNT and CDs are composed of two cell populations, the principal cells and the intercalated cells that are further separated in distinct populations (α and β). Different collecting ducts branches join to form the cortical collecting tubule (CCT) which are divided to outer and inner medullary CD (OMCD and IMCD, respectively) with only one cell type present (Boron & Boulpaep, 2005).

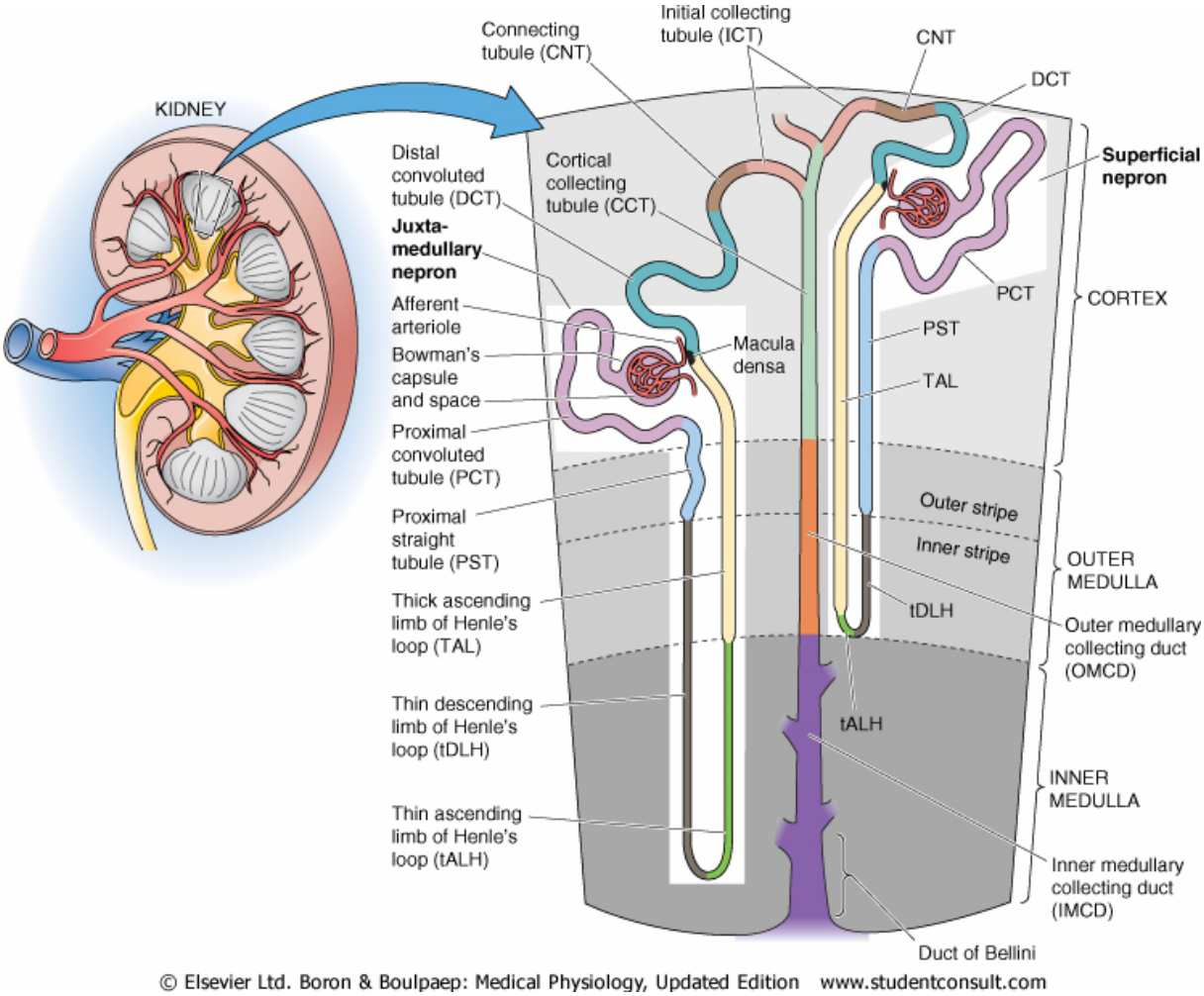

Figure 4. Nephron structure (Boron & Boulpaep, 2005).

5. Amino acid transporters

5.1 Membrane Transport of Small Molecules

As mentioned earlier, the transcellular transport of small water-soluble molecules like ions, sugars and amino acids is limited by the hydrophobic lipid bilayer of the membrane. Specific sets of transport proteins allow uptake and efflux of these solutes to occur.
Membrane transport proteins are typically classified in pores, carriers and channels. Pores are always open structures (e.g. aquaporin). Carriers are never fully open and undergo conformational changes upon binding of a solute to a carrier on one side of the membrane that leads to the release of the solute on the other side of the membrane (e.g. amino acid transporters, glucose transporters). This is in contrast to channels that are alternatively open and closed. Though they have weaker interactions with the solutes, they form aqueous pores in the open conformation (e.g. ion channels). The formation of a pore implies that the rate of transport of channels is much faster than that of carriers.

Channels only mediate the passive transport of solutes, in contrast to carriers that may mediate passive transport (or facilitated diffusion). In that case, the solute follows its electrochemical gradient. Carriers may mediate passive or active transport, the latter being coupled to an energy source.
Carrier mediated solute transport is similar to an enzyme-substrate reaction. The carrier can be saturated up to a maximum transport rate V_{max}, which depends on the speed of the conformation change. Each solute also has a specific binding affinity to the carrier (K_m) that is the concentration of solute when the transport is half of V_{max}.
Carriers may be uniporters if only a single solute is transported, or coupled transporters if more than one solute is transported. The transporter is then described as a symporter when the coupled transport is in the same direction or as an antiporter when solutes move in opposite directions.
The energy necessary for active transport against an electrochemical gradient in animal cells may directly or indirectly come from ATP hydrolysis leading to the classification of primary, secondary or tertiary active transporters. In primary active transport solute movement against its electrochemical gradient is associated to the hydrolysis of ATP. Secondary active transport by a coupled transporter uses the gradient generated by the primary active transport and couples it to a solute's movement against an electrochemical gradient. Tertiary active transport functions similarly. For example, the Na/K-ATPase is a primary active transporter that extrudes Na^+ and imports K^+ against their electrochemical gradients and that is fuelled by ATP hydrolysis. Na^+ is then prone to enter the cells coupling its transport to, for

example, dicarboxylates via the secondary active Na^+ dicarboxylate transporter NaDC1. The generated dicarboxylate gradient can then be used by the tertiary active dicarboxylate / organic anion transporter (OAT1) to import organic anions inside the cells (Alberts et al, 2002; Boron & Boulpaep, 2005).

5.2 Amino Acid Transporters

In 1913, Van Slyke and Meyer showed that amino acids are absorbed from the blood to the tissues against a concentration gradient by infusing digested casein in the blood of dogs resulting in 5-10 times more amino nitrogen in the tissues than in blood (Van Slyke & Meyer, 1913). In the 1960s, amino acid transport was investigated in erythrocytes, hepatocytes (Kilberg et al, 1980), fibroblasts and other cell types (Christensen & Oxender, 1960) and classified in systems. These systems transport amino acids depending on their physico-chemical properties, stereospecificity, size and charge. Further characterisation of the systems included Na^+ and pH dependency, inhibition by synthetic amino acids, and showed that some systems had overlapping functions (Christensen, 1989).

An attempt to simplify the nomenclature was suggested in 1984. The guideline was to use roman letters that would not evoke a single amino acid since these systems usually transport multiple amino acids. The symbols y^+, x^- and ^{0} were proposed for basic, acidic and zwitterionic amino acid transporter systems with the possibility to add subscript letters to indicate specificity (though it does not follow the single letter code for acidic amino acid systems A: asparate, G: glutamate, C: cystine). Na^+ dependency is usually indicated by capitalisation though the previously characterised Na^+-independent system L (referring to its preferred amino acid leucine) was not retroactively modified (Bannai et al, 1984; Oxender & Christensen, 1963).

Genetic identification of the molecular correspondents of amino acid transport systems began in the early 90's (Broer, 2008; Kanai & Hediger, 1992; Kavanaugh et al, 1994; Palacin et al, 1998; Verrey et al, 1999) and usually followed the systems nomenclature with the addition of AT for amino acid transporter, as well as a number for different variants sharing a same transport system (e.g. system L: LAT1, LAT2, …). This allowed functional characterisation of the transporters in cells or in the *Xenopus laevis* oocytes heterologous system, the measurement of mRNA expression levels in tissues and the production of antibodies for localisation studies. Further investigations of the transporters' physiological impact *in vivo* were investigated in knock-out mouse models (Peters et al, 2003; Quan et al, 2004; Singer et al, submitted; Sperandeo et al, 2007; Stoffel et al, 2004; Tsumura et al,

2003). An overview of the different amino acid transporters and corresponding systems is displayed in table 4.

Transport system	Transporters (associated with)	Other names	Gene	Mechanism	Physiological substrates[1]
System A	SNAT1	ATA1, GlnT, SAT1, NAT2, SA2	SLC38A1	$1Na^+/AA^2$ cotransport	G, A, S, C, Q, N, H, M
	SNAT2	ATA2, SA1, SAT2	SLC38A2		G, P, A, S, C, Q, N, H, M
	SNAT4	ATA3, NAT3, PAAT, SAT3	SLC38A4		G, P, A, S, C, N, M
System asc	Asc1 (4F2hc)	-	SLC7A10	antiporter	Small neutral D and L-AA
	Asc2 (?hc)	-	SLC7A-		G, A, S, T
System ASC	ASCT1	SAAT1	SLC1A4	Na^+-dependent Or antiporter	A, S, C
	ASCT2	ATB^0	SLC1A5		A, S, C, T, Q
System $b^{0,+}$	$b^{0,+}AT$ (rBAT)	NBAT/lc6	SLC7A9	antiporter	Cationic and large neutral AA
System B^0	B^0AT1 (Tmem27/ACE 2)	XT2s1	SLC6A19	Na^+/AA cotransport	Broad range neutral AA
	B^0AT2	NTT v3-7	SLC6A15		P, Large neutral AA
	B^0AT3 (Tmem27)	XT2	SLC6A18		Broad range neutral
System $B^{0,+}$	$ATB^{0,+}$	-	SLC6A14	$2Na^+/1Cl^-$/AA cotransport	Neutral and cationic AA
System β	GAT1	-	SLC6A1	Na^+/Cl^-/AA cotransport	GABA
	GAT2	BGT1	SLC6A12		GABA, betaine
	GAT3	GAT2	SLC6A13		GABA
	GAT4	GAT3	SLC6A11		GABA
System Gly	GlyT1	-	SLC6A9	$2\text{-}3Na^+/1Cl^-$/AA cotransport	G
	GlyT2	-	SLC6A5		G
Imino acid carrier	PAT1	LYAAT1	SLC36A1	$1H^+$/AA cotransport	P, G, A, β-Ala, GABA
	PAT2	Tramdorin1	SLC36A2		P, G, A

Transport system	Transporters (associated with)	Other names	Gene	Mechanism	Physiological substrates[1]
System IMINO	SIT1 (Coll/ACE2)	XT3s1, XT3	SLC6A20	Na^+/Cl^-/AA cotransport	Imino acids
System L	LAT1 (4F2hc)	-	SLC7A5	antiporter	Large neutral AA
	LAT2 (4F2hc)	4F2hc-lc5	SLC7A8		Neutral AA
	LAT3	-	SLC43A1	uniport	Neutral AA
	LAT4	-	SLC43A2		Neutral AA
System N	SNAT3	NAT1, SN1	SLC38A3	Na^+/AA cotransport/H^+-antiport	Q, N, H
	SNAT5	SN2	SLC38A5		Q, N, H, S, G
System T	TAT1	MCT10	SLC16A10	uniport	F, Y, W
System X_{AG}	EAAT1	GLAST	SLC1A3	$3Na^+/1H^+$/AA cotransport/$1K^+$-antiport	E, D
	EAAT2	GLT-1	SLC1A2		E, D
	EAAT3	EAAC1	SLC1A1		E, D, C
	EAAT4	-	SLC1A6		E, D
	EAAT5	-	SLC1A7		E, D
System x^-_c	xCT (4F2hc)	-	SLC7A11	antiporter	E, C- (no D)
System y^+	CAT-1	ATRC1	SLC7A1	uniporter	R, K, H
	CAT-2A/B	ATRC2, TEA	SLC7A2	?	R, K, H
	CAT-3	ATRC3	SLC7A3		R, K
	(CAT-4)	-	SLC7A4		?
System y^+L	y^+LAT1 (4F2hc)	-	SLC7A7	Na^+-dependent antiporter	K, R, Q, H, M, L
	y^+LAT2 (4F2hc)	-	SLC7A6		K, R, Q, H, M, L, A, C

Table 4. Overview of amino acid transporter systems, corresponding proteins, genes and substrates. [1]Amino acids are given in the one-letter code (C- Cystine), weak substrates are shown in parenthesis. [2]AA: amino acids. Adapted from (Broer, 2008).

5.3 The SLC6 family

Transporters or Solute Carriers (SLC) of the human genome are organised in 43 families with 20-25% of amino acid sequence identity within a family (Hediger et al, 2004).
The sodium- and chloride-dependent neurotransmitter and amino acid transporter family SLC6 is, with 20 members, the largest solute carrier family of transporters. SLC6 members code for proteins of approximately 600 amino acids with 12 transmembrane segments and intracellular N- and C- termini (Nelson, 1998). A specific feature of this family is a large extracellular loop between transmembrane segments 3 and 4. It has also been shown that some members may form homo-oligomers. Transport of solutes against the electrochemical gradient is driven by Na^+ co-transport with the number of co-transported Na^+ varying from 1 to 3. In many cases, the co-transport of Cl^- also occurs (Broer, 2006; Chen et al, 2004).

The phylogenetic tree of this family evidences four distinct branches (Figure 5):

- The GABA transporter branch with the γ-aminobutyric acid (GABA) transporter 1 (GAT1, Slc6a1), the first member to be identified and extensively characterised. This cluster also contains the creatine transporter 1 (CT1, Slc6a8) and the taurine transporter (TauT, Slc6a6).
- The monoamine transporter branch with the dopamine (DAT, Slc6a3), the serotonin (5HTT, Slc6a4) and the norepinephrine (NET, Slc6a2) transporters.
- An amino acid branch, containing β-alanine, neutral and cationic amino acid transporter $ATB^{0,+}$ (Slc6a14) as well as glycine (GlyT1/2, Slc6a9/5) and proline transporters (ProT, Slc6a7) predominantly expressed in brain (Eulenburg et al, 2005).
- The transporters of the second branch of amino acids, previously known as the “orphan transporters” branch will be described further in sections 5.3.1-5.3.3. They have been shown to localise on the apical membrane of kidney proximal tubule, and in some cases small intestine. Correct localisation at the membrane is dependent on co-expression of tissue specific associated proteins Tmem27 in the kidney and homolog ACE2 in the small intestine (see section 6.2.1-6.2.2) (Camargo et al, in press; Danilczyk et al, 2006; Malakauskas et al, 2007; Singer et al, submitted).

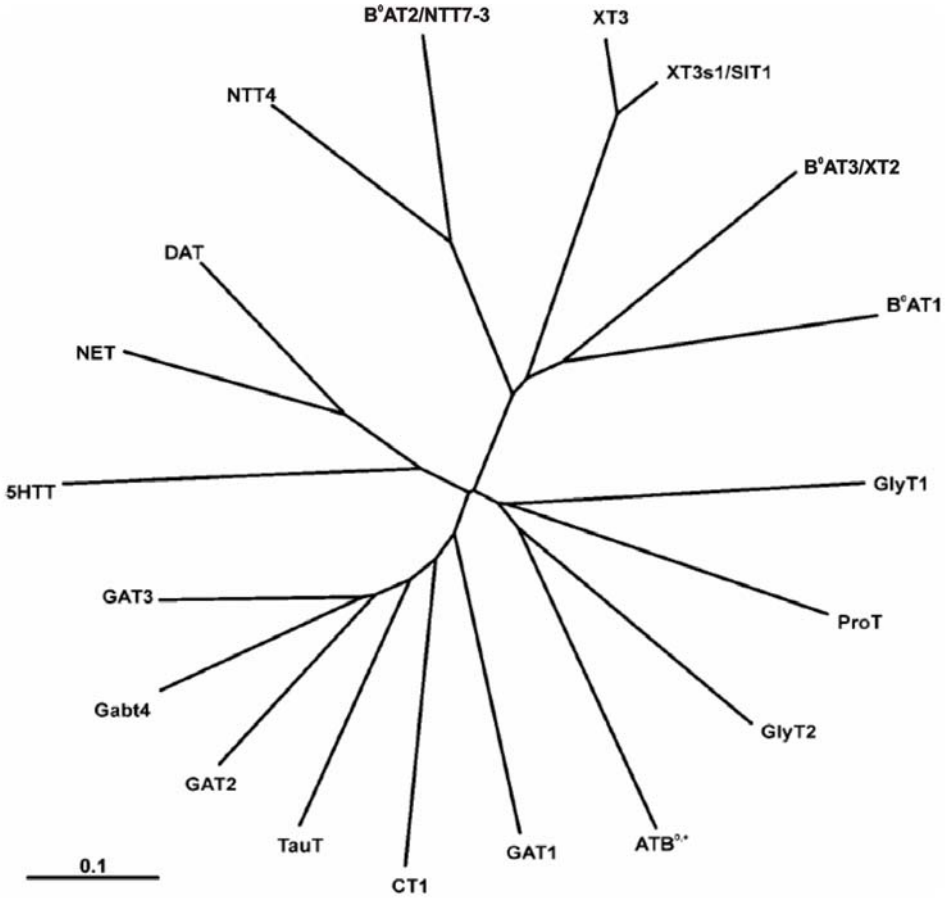

Figure 5. Phylogenetic tree of the mouse SLC6 family (Romeo et al, 2006).

5.3.1 B^0AT1 (Slc6a19)

Transport of large neutral amino acids in kidney and in small intestine was first described to be controlled by system B^0 (Doyle & McGivan, 1992a; Doyle & McGivan, 1992b). Evidence of B^0AT1 transporter involvement came through the characterisation of the autosomal recessive Hartnup disorder. This disorder is characterized by neutral aminoaciduria due to impaired amino acid transport in kidney proximal tubule whereas the extent of intestinal transport impairment appears to be less consistent. Various other clinical symptoms such as pellagra-like rash, cerebellar ataxia and other neurological dysfunctions may be present in affected individuals, while other subjects remain asymptomatic besides aminoaciduria (Baron et al, 1956). Phenotypic variability might be partially explained by the differential impact of various mutations and the frequent compound heterozygosity. Little was known about the cause of the disorder but its link to chromosome 5p15 in human (Nozaki et al, 2001). Screening for genes encoding proteins with more than 5 transmembrane domains typical of the SLC

members identified genes Slc6a18 (B^0AT3) and Slc6a19 (B^0AT1). In mouse, the slc6a19 gene localises to chromosome 13 in cytoband C1. It has 12 exons encoding a 634 amino acids protein (Bohmer et al, 2005; Camargo et al, 2005).

Expression of mouse Slc6a19 in the *Xenopus laevis* oocytes heterologous system induced electrogenic transport of a broad range of neutral amino acids. In that system, B^0AT1 has a low affinity constant for L-leucine ($K_{0.5}$ = 1.16 mM) (Bohmer et al, 2005; Camargo et al, 2005). Additionally, B^0AT1 substrate-induced currents are Na^+-dependent and Cl^-- and H^+-sensitive. In accordance with the *in vitro* data, B^0AT1 localises to the brush border membrane on the apical side of villi enterocytes in the small intestine and early proximal tubule cells (S1, S2) in the kidney (Romeo et al, 2006). Proximal tubule reabsorption of solutes often displays axial gradient of distribution of a low affinity component in the early segments (S1) and a higher affinity component in the later segments (S3). Accordingly, glucose and peptide transporters SGLT2 (Slc5a2) and PepT1 (Slc15a1) in S1 have a complementary distribution to that of SGLT1 (Slc5a1) and PepT2 (Slc15a2). For neutral amino acids, B^0AT1 probably corresponds to the low affinity component, while B^0AT3 (see section 5.3.2) corresponds to the high affinity component (Anderson et al, 2004; Boll et al, 2004; Daniel et al, 2006).

In contrast to mouse B^0AT1, *X. laevis* oocytes expressing human B^0AT1 only accumulated little amounts of radiolabeled amino acids, and the current generated by the co-transport of Na^+ and amino acid were too small to characterise (~ 10 nA). This suggested that correct expression of the human B^0AT1 at the plasma membrane required co-expression of an associated protein. The work presented in this thesis reports the organ specific association of B^0AT1 with Tmem27 in the kidney and ACE2 in the small intestine (Camargo et al, in press; Danilczyk et al, 2006; Malakauskas et al, 2007) (see sections 6.1.2 and 6.1.3). Indeed, co-expression of the associated proteins in oocytes confirmed human B^0AT1 functional correspondence to the mouse ortholog. The relationship between Hartnup disorder and B^0AT1 was confirmed when most cases of the disorder were shown to be caused by mutations in B^0AT1 (Kleta et al, 2004). We also showed that Hartnup disorder-causing mutations differentially interact with the associated proteins, thereby participating in phenotypic heterogeneity (Camargo et al, in press).

5.3.2 B^0AT3 / XT2 (Slc6a18)

The B^0AT3 gene Slc6a18, previously known as orphan transporter XT2, is arranged in tandem with that of B^0AT1 (Slc6a19) on chromosome 5 in human and 13 in mouse (Nash et al, 1998). These genes share 52% sequence identity and thus presumably arose by gene duplication (Romeo et al, 2006). Mouse Slc6a18 was identified by screening a genomic library with a rat dopamine transporter-derived probe with a highly conserved motif. Slc6a18 is constituted of 13 exons with alternative splicing and a splice acceptor, resulting in 6 transcribed isoforms. The A12 isoform is however the only one with 12 putative transmembrane segments, in accordance with the SLC6 family members organisation. The physiological significance of the five shorter transcripts has until now not been established (Nash et al, 1998).

The rat ortholog of Slc6a18 is a renal osmotic stress-induced transcript (ROSIT) which was first identified as an upregulated transcript in hypertonic renal cortex (Wasserman et al, 1994). However, study of mouse B^0AT3 in osmotic stress conditions did not verify such a regulation (Elisa Romeo and François Verrey, unpublished data). Already then, the function of ROSIT, and later of mouse Slc6a18 (94.6% identity) was tested in *X. laevis* oocytes without success (Nash et al, 1998; Quan et al, 2004; Wasserman et al, 1994). Direct function of B^0AT3 eluded demonstration for 10 years, until co-expression with associated protein ACE2, but not Tmem27, in *X. laevis* oocytes made characterisation possible. As hypothesised based on its high identity to B^0AT1, B^0AT3 transports a broad range of neutral amino acid (A, M, V, I > G, S, L). Further characterisation showed that L-isoleucine is transported with high affinity ($K_{0.5}$ = 0.21 mM) in a Na^+ and Cl^- dependant as well as pH-independent manner (Singer et al, submitted). In contrast to the association with ACE2 observed in *Xenopus laevis* oocytes, experiments with *ace2* and *tmem27* null mice demonstrate that *in vivo* it is Tmem27, a smaller homologue of ACE2, which is required for functional expression of B^0AT3 in kidney (Danilczyk et al, 2006; Malakauskas et al, 2007; Singer et al, submitted).

Localisation studies performed in mouse tissues have shown that B^0AT3 is highly expressed in kidney, where it localises to the brush border membrane of the late proximal tubule (S2, S3), in a complementary fashion to B^0AT1 that localises to the early proximal tubule (S1) (Romeo et al, 2006). As previously mentioned, B^0AT3 corresponds to the high affinity component of neutral amino acid transport.

An *Slc6a18* null mouse model was first generated and analyzed by the group of Marc Caron. The high amounts of glycine found in the urine of these mice supported the hypothesis that the orphan gene product B^0AT3 functioned as an amino acid transporter. Brush border membrane vesicles uptake additionally evidenced its function as a high affinity transport

system of glycine (Quan et al, 2004). The work presented in this thesis re-analysed the B^0AT3 knock-out mouse model after more than 10 generations of backcrossing into C57BL/6 background. Next to the previously published glycinuria, a urinary loss of several other amino acids, in particular beta-branched and small neutral ones was observed.

Surprisingly, the *Slc6a18* null mice displayed a systolic blood pressure that was 15 - 20 mmHg higher than that of their wild-type litter mates, a difference that was abolished upon glycine supplementation in drinking water. Using telemetry, the previously described link of B^0AT3 lack and hypertension was confirmed, but only in physically restrained animals (Singer et al, submitted). Such an impact of B^0AT3 on blood pressure was not confirmed in human where a SNP within the SLC6A18 gene present in 46.7% of a general Japanese population corresponds to a nonsense mutation (Y319X) and is not associated with hypertension (Eslami et al, 2006).

5.3.3 SIT1 / XT3s1 / XT3 (Slc6a20)

The human sodium / imino-acid transporter SIT1 (XT3, Slc6a20) corresponds to system IMINO, mediating proline transport and escaping alanine inhibition (Stevens & Wright, 1985; Stevens & Wright, 1987). This gene localises to chromosome 3p21.3, is transcribed to a 592 amino acids protein and is expressed in small intestine epithelium and kidney (Takanaga et al, 2005). An additional 555 amino acids form that arises following the deletion of a transmembrane segment by alternative splicing is present in brain (Kiss et al, 2001).

Nomenclature in mouse may lead to possible confusions as there are two closely related but distinct genes on chromosome 9F4 (90% identity), Slc6a20a ("similar to XT3" XT3s1) and Slc6a20b (XT3). Characterisation of murine Slc6a20 variants in *X. laevis* oocytes showed that XT3s1/SIT1 corresponds to SIT1, whereas orphan XT3 expression alone did not lead to any uptake of the tested amino acid substrates (Kowalczuk et al, 2005). XT3s1 has been found to localise to the brush border of mouse kidney proximal tubule and small intestine enterocytes, and also has a broad tissue expression (brain (cerebellum, cortex, brain stem), thymus, spleen and lung) (Romeo et al, 2006; Takanaga et al, 2005). XT3 is only found expressed in kidney with a localisation similar to that of XT3s1 (Romeo et al, 2006). Similarly to B^0AT1, expression of XT3s1 may be controlled by the associated proteins Tmem27 in the kidney and possibly ACE2 in the small intestine (Danilczyk et al, 2006).

Study of human and mouse SIT1 showed high affinity uptakes of L-proline (K_m = 0.17 mM and 0.13 mM respectively), MeAIB and betaine, typical of IMINO system. Additionally,

susbtrate transport is electrogenic and displays Na^+ and Cl^- dependence with a stoichiometry of 1:2:1 (Kowalczuk et al, 2005; Takanaga et al, 2005).

Other entry routes for L-proline include the imino acid carrier PAT1 that additionally transports glycine as well as B^0AT1 (Anderson et al, 2004; Boll et al, 2004; Camargo et al, 2005; Munck et al, 1994; Roigaard-Petersen et al, 1987).

6. Regulation of Blood Pressure

6.1 General

The regulation of blood pressure on a short and long term time scale is necessary to appropriately perfuse organs in response to the different demands of everyday life.

Short term regulation mainly consists of a neural reflex composed of a series of negative feedback loops with stretch receptors or mechanoreceptors in the carotid sinus and the aortic arch serving as detectors of blood pressure variation. These variations are then transduced to an electrical signal which is transmitted to the coordinating centre in the central nervous system through afferent neural pathways. Most of the information is processed in the nucleus tractus solitarius in the medulla oblongata with excitatory projections to the cardioinhibitory area or inhibitory projections to the vasomotor area. Sympathetic and parasympathetic efferent neural pathways to effectors in the heart (pacemaker cells and muscle cells), the veins (vascular smooth muscle cells, VSMCs) and the adrenal medulla (chromaffin cells) accordingly regulate blood pressure.

Long term control of blood pressure (hours – days) is modulated by the humoral control of vessels and kidney function either affecting cardiovascular and VSMCs (vasoactive) or control of extracellular fluid volume (ECF, non-vasoactive). Vasoactive effectors include angiotensin II (Ang II), bradykinin, epinephrine, serotonin, and histamine. Non-vasoactive effectors control ECF volume through Na^+ balance control. As no sensor measures a volume, the fullness of the system can be sensed through several pathways: the previously mentioned high and low pressure sensors of the autonomic nervous system, the renin angiotensin aldosterone system (RAAS, see section 6.1), the atrial myocytes secreting the atrial natriuretic peptide (ANP) and the osmoreceptors in the central nervous system that stimulate thirst and release of the antidiuretic hormone arginine vasopressin (AVP) by the posterior pituitary (Figure 6) (Boron & Boulpaep, 2005).

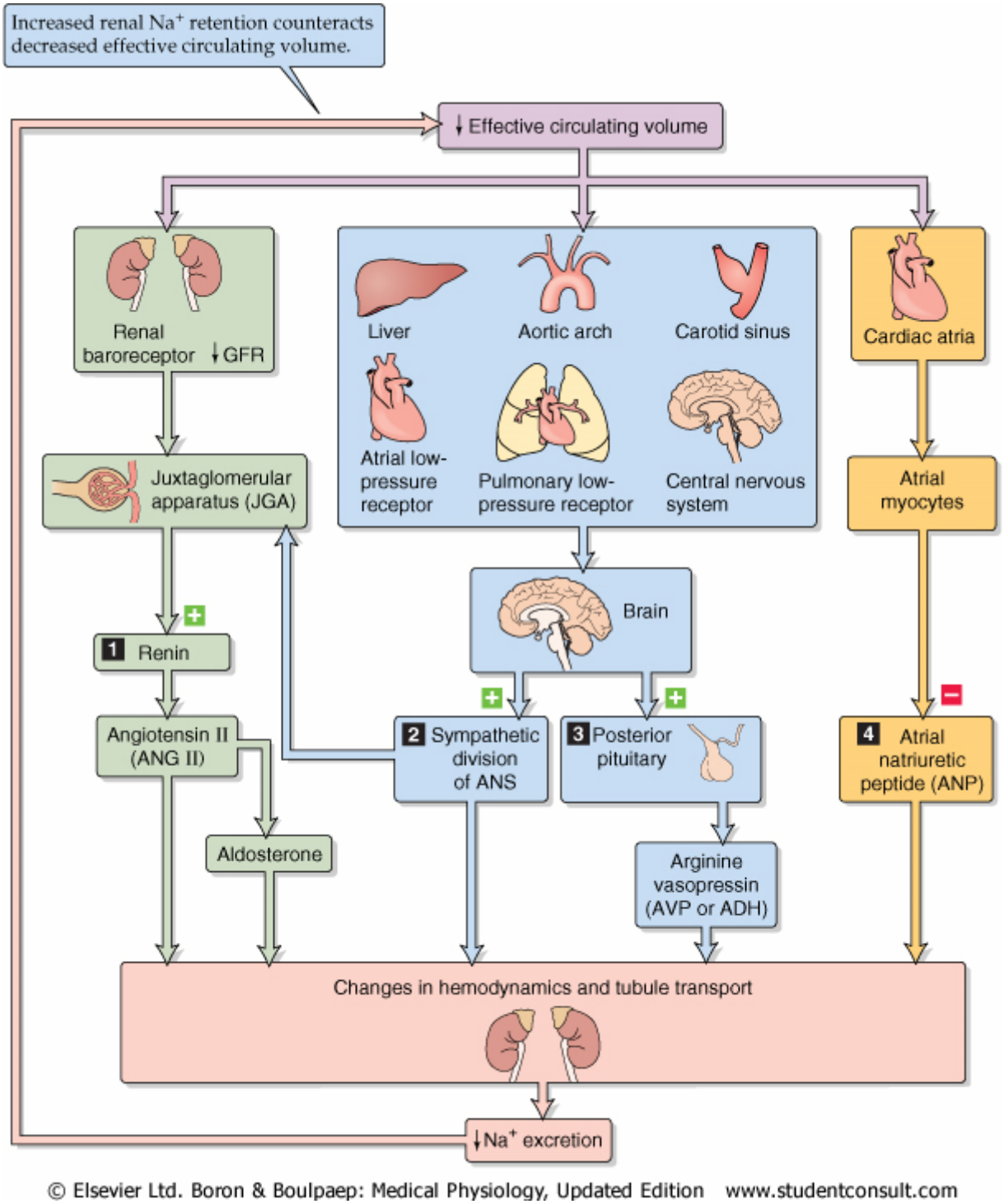

Figure 6. Feedback control of effective circulating volume. A low effective circulating volume triggers four parallel effector pathways that act on the kidney, either by changing hemodynamics or by changing Na^+ transport by the renal tubule. ANS: autonomic nervous system. GFR: glomerular filtration rate (Boron & Boulpaep, 2005).

6.2 The Renin Angiotensin Aldosterone System

The renin angiotensin aldosterone system (RAAS) controls blood pressure through the release of angiotensin II (Ang II). Renin is first released by the granular cells of the juxtaglomerular apparatus in the kidney in reponse to low effective circulating volume. This can be sensed by different systems: sympathetic stimulation following a decrease in systemic blood pressure, decrease in NaCl concentration at the macula densa, decrease in renal perfusion pressure or decrease in the input to the stretch receptors of the granular cells.

Renin release in the circulation cleaves the decapeptide precursor angiotensinogen synthesised and released by the liver to the octapeptide Ang I. Ang I is converted by the dipeptidyl carboxypeptidase angiotensin-converting enzyme (ACE) to Ang II, the main effector peptide of the RAAS. Ang II displays potent vasoconstrictive, pro-inflammatory, and pro-fibrotic properties. The majority of the cardiac and renal actions of Ang II, including vascular smooth muscle contraction, aldosterone secretion, adrenergic stimulation and renal sodium reabsorption are mediated by the AT1 receptor. Ang II also binds to AT2 receptors, inducing a counter regulatory vasodilation that is largely mediated by bradykinin and nitric oxide (NO, see section 6.3) (Figures 7 and 8).

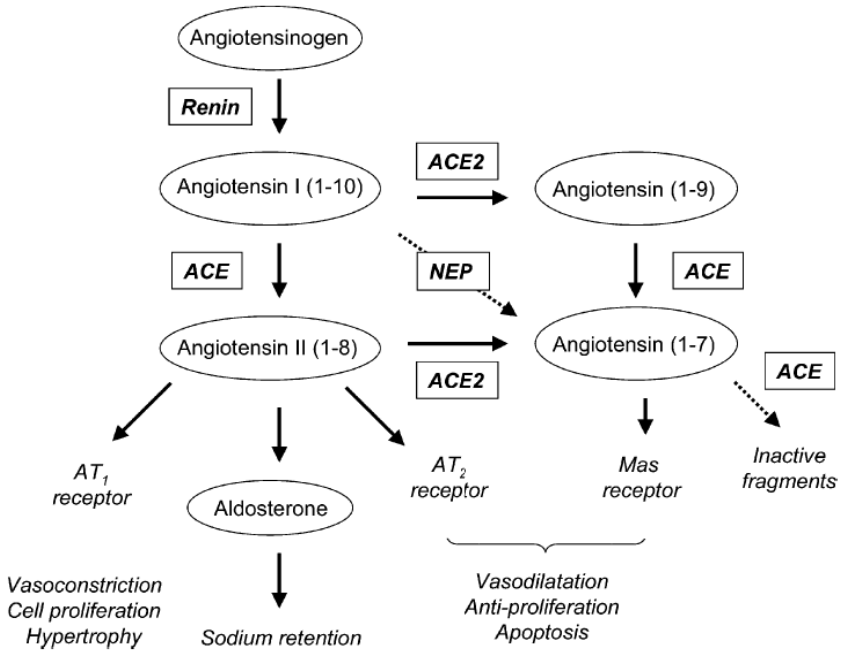

Figure 7. Schematic diagram of the renin angiotensin aldosterone system which shows the role of ACE and ACE2 in the metabolism of the various angiotensin peptides (Hamming et al, 2007).

The main role of Ang II is the control of Na^+ reabsorption through different mechanisms. A stronger constriction of the efferent arteriole compared to the afferent arteriole leads to enhanced Na^+ reabsorption, the blood flow through the vasa recta is reduced preventing NaCl and urea to be washed-out of the medulla, the sensitivity of the tubuloglomerular feedback increases and the Na^+/H^+ exchanger actitivity is enhanced. Ang II also stimulates the release of aldosterone by the glomerulosa cells in the adrenal cortex, which stimulates Na^+ reabsorption in collecting tubules. Ang II additionally stimulates thirst and AVP secretion in the central nervous system (Boron & Boulpaep, 2005) (Figure 8).

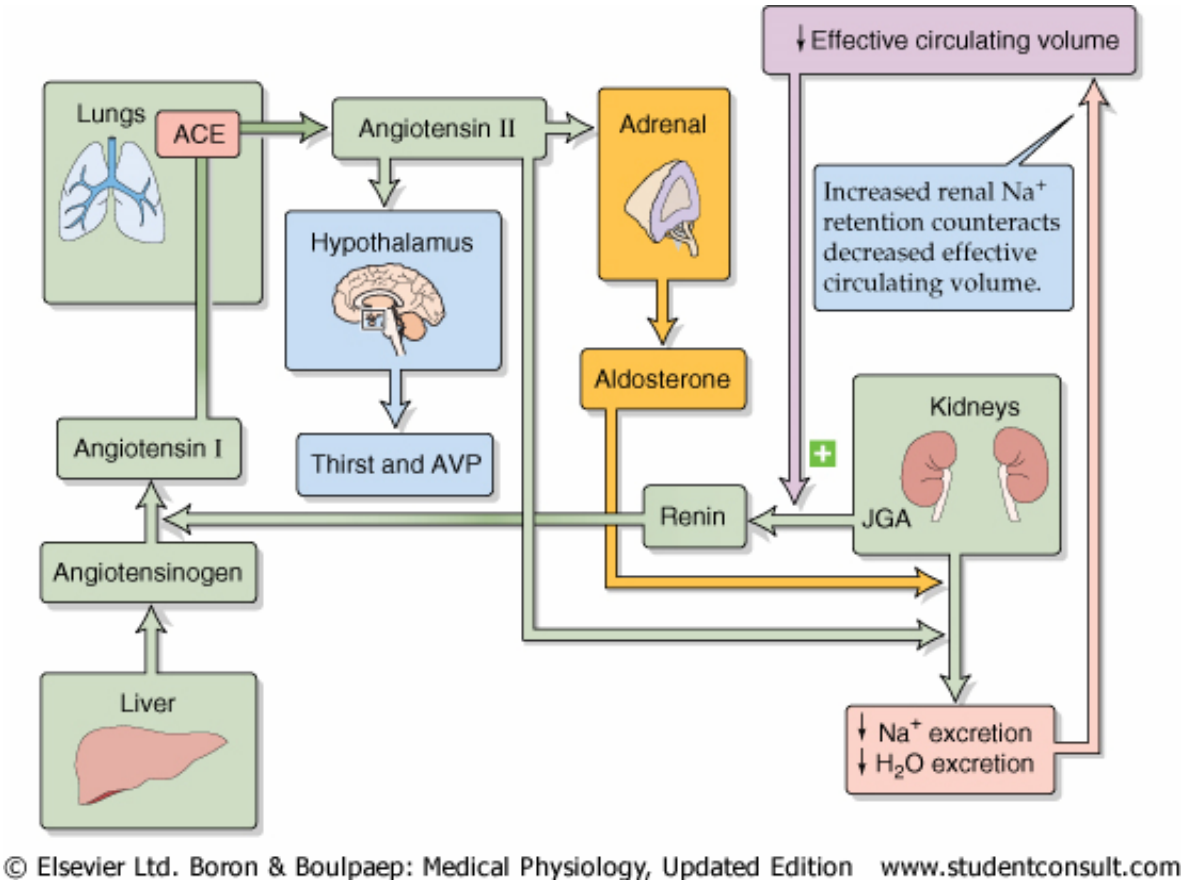

Figure 8. The renin angiotensin aldosterone system (Boron & Boulpaep, 2005).

The complexity of the classical RAAS regulation of blood pressure was increased with the identification of new enzymes regulating the system's peptides, such as the angiotensin-converting enzyme 2 (ACE2) and the presence of local RAS.

6.2.1 ACE2 / ACEH

ACE2 (ACEH), a homolog of ACE is an essential carboxypeptidase of the RAS that primarily cleaves Ang II to Ang (1-7), but also Ang I to Ang (1-9). ACE2 action reduces Ang II concentration and counterbalances the action of ACE. Additionally, it has been shown that Ang (1–7) mediates vasodilatation, antiproliferation, and apoptosis, thereby opposing the effects of Ang II (Figure 7).

ACE2 was originally identified in two independent studies, a screen for new metalloproteases (Tipnis et al, 2000) and a human heart failure ventricle library (Donoghue et al, 2000). It is located in the Xp22 locus, contains 18 exons encoding an 805 amino acids type I glycoprotein with a 17 amino acids signal peptide and a single transmembrane segment (Figure 9). The single HEMGH zinc-binding catalytic domain is encoded by exon 9.

ACE2 localises to the apical membrane in polarized cells and is downregulated after extracellular shedding by the ADAMs family of zinc metalloproteinases (Lambert et al, 2005), and by binding of the SARS coronavirus (Imai et al, 2005; Kuba et al, 2005).

ACE2 recognises the consensus sequence *Pro-(1-3 residues)-Pro-hydrophobic amino acid* cleaving the terminal residue of a variety of peptides such as neurotensin-(1–11), dynorphin A-(1–13) (endogenous opioid), β-casomorphin-(1–7), ghrelin and apelin (13 and 36) but not bradykinin (Vickers et al, 2002).

ACE2 was originally described as being expressed in kidney, heart and testis, but further investigation identified it in a variety of tissues such as VSMCs, lung, liver, small intestine, placenta and brain (Hamming et al, 2007).

Three *ace2* null mice models were generated by deleting the catalytic domain encoding exon 9. As expected from the role of ACE2 in the RAS, the three models led to Ang II increase in kidney, heart and plasma as measured by all three groups. The role of ACE2 on heart function and blood pressure regulation was investigated due to the expression of ACE2 in endothelial cells, smooth muscle cells from intra-myocardial vessels, and cardiac myocytes, but the findings were not consistent and remain inconclusive (Crackower et al, 2002; Gurley et al, 2006; Yamamoto et al, 2006). The first *ace2* knock-out mouse model generated by the group of Joseph Penninger confirmed the importance of this protein in heart function and morphology. These *ace2* null mice had a slight wall thinning of the left ventricle and increased heart chamber dimension. This led to a reduction in cardiac contractility and was primarily observed in 6 months old male mice. The authors hypothesise that increased Ang II levels are responsible for the heart phenotype as it is rescued by additionally knocking the *ace* gene out. This cardiac defect was not observed in the *ace2* null mice independently

generated by the group of Thomas Coffman, or by the group of Toshio Ogihara. A cardiac phenotype was however still evidenced as *ace2* null mice had lower heart rates in 3 and 6 months old mice (Gurley et al, 2006) and an accelerated pressure overload-induced cardiac dysfunction from increased local Ang II levels (Yamamoto et al, 2006).
Since the RAAS controls blood pressure and ACE2 localises to the vascular endothelial cells and smooth muscle cells, disruption of the ACE2 component was hypothesised to lead to hypertension (Hamming tissue distribution ACE2 2004). Though ACE2 mRNA is downregulated in hypertensive rats (Crackower et al, 2002), comparison of baseline blood pressures in the different *ace2* knock-out mice models was, similarly to the heart phenotype, inconsistent between the different models. A first model only showed hypotension due to the decreased cardiac contractility in 6 months old *ace2* null mice (Crackower et al, 2002). In the second model, there was a slight hypertension in *ace2*$^{-/y}$ in a C57/Bl6 background of about 7 mmHg. Chronic Ang II administration increased blood pressure in these mice twice as much as in the wild-type controls. Interestingly, the blood pressure of *ace2* null mice back-crossed in a 129/Sv exhibited no difference when compared to *ace2* wild-type littermates whether chronically treated with Ang II or not, showing the importance of the background strain when measuring blood pressure (Gurley et al, 2006).

The kidney is another important organ implicated in the regulation of blood pressure and where ACE2 is highly expressed. In the kidney, ACE2 is expressed on the apical side of the proximal tubule with an axial gradient towards the earlier segments (Singer et al, submitted), in smooth muscle cells of renal vessels (Hamming et al, 2007), and in glomerular visceral and parietal epithelial cells. Angiotensinogen is directly secreted into the tubule lumen, where it serves as a substrate for renin or renin-like enzymes increasing local renal interstitial fluid Ang II levels to a 1000 times more than in plasma. A disrupted balance between intrarenal ACE and ACE2 with consequent high levels of Ang II contributes to progressive renal damage. Accordingly, *ace2* null mice develop a late onset of glomerulosclerosis (Oudit et al, 2006) and renal ACE2 expression is decreased in experimental hypertension and diabetes (Crackower et al, 2002; Tikellis et al, 2003).

Additionally, ACE2 and possibly Ang II levels in general have surprising effects on amino acid and glucose homeostasis. As previously mentioned, ACE2 and its smaller homologue Tmem27 control amino acids transporters expression in the small intestine and in the kidney, respectively (see sections 5.3.1-5.3.3 and 6.2.2). The work presented in this thesis shows the physiological impact of the lack of certain intestinal amino acid transporters in *ace2* null mice (see section 9). Though no mechanism has been suggested, glucose homeostasis also seems to be impaired in *ace2* null mice that display selective decrease in first-phase insulin

secretion in response to glucose and a progressive impairment of glucose tolerance (Niu et al, 2008).

Finally, the general intestinal RAS system has also been shown to regulate sodium-dependant glucose transporter 1 (SGLT1) expression in rat small intestine. There, SGLT1-mediated glucose transport in intestinal ring segments was decreased by exposure to Ang II through transporter downregulation, which was reversible by AT1 receptor antagonist treatment (Wong et al, 2007).

Furthermore, ACE2 is not only involved in cardiovascular and renal (patho)physiology, but also in diabetes, acute lung disease and pregnancy (Hamming et al, 2007).

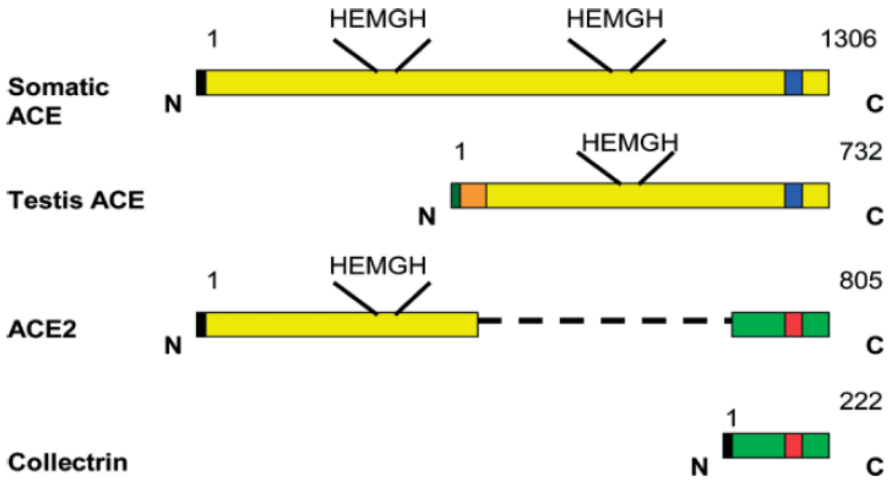

Figure 9. The domain structure of somatic and testis ACE, ACE2, and Tmem27 (Collectrin). Each protein is a type I integral membrane protein with a cleaved signal peptide (black), an N-terminal ectodomain, a transmembrane domain (blue in ACE; red in ACE2 and Collectrin), and a C-terminal cytoplasmic domain. HEMGH: zinc binding active site (Hamming et al, 2007).

6.2.2 Tmem27 / Collectrin

Tmem27, or Collectrin, is a homolog of ACE2, sharing 47.8% identity with its non-catalytic extracellular, transmembrane and cytosolic domains, but does not contain the catalytic activity domain (Figure 9). ACE2 and Tmem27 constitute an ancient gene duplication that are always located in close proximity on the same chromosome throughout evolution (Chou, gene, 2006).

Tmem27 was originally described as dramatically upregulated in 5/6 nephrectomized mouse kidney (NX-17) in a screen to identify genes involved in glomerular hyperfiltration and hypertrophy (Zhang et al, 1999).

The *tmem27* gene localises next to ACE2 on chromomsome Xp22 and encodes a 222 amino acid protein with a signal peptide that represents a 32 kDa membrane glycoprotein with a single transmembrane segment (Zhang et al, 2001). It was originally described to specifically localise to the kidney collecting duct membrane and cytosol, hence the name Collectrin. Further studies showed a broader expression in liver, lung, endocrine pancreas and kidney proximal tubule brush border membrane where it is necessary for SLC6 amino acid transporters expression (Akpinar et al, 2005; Danilczyk et al, 2006; Malakauskas et al, 2007; Stoffel et al, 2004). As mentioned earlier, *tmem27* null mice exhibit a dramatic decrease of SLC6 amino acid transporters in kidney proximal tubule that leads to massive aminoaciduria (Danilczyk et al, 2006; Malakauskas et al, 2007).

The promoter of *tmem27* is under the control of both hepatocyte nuclear factor 1 α and β (HNF1α and HNF1β). Indeed, a decrease of Tmem27 expression in the *hnf1*α null mouse model is probably responsible for the aminoaciduria component of the general defect of proximal tubule reabsorption in these mice (Fanconi syndrome) (Pontoglio et al, 1996). The implication of Tmem27 with the maturity onset diabetes of the young (MODY) was then investigated, as mutations in transcription factors HNF1α and HNF1β are responsible for this disease along with mutations in the gene encoding glucokinase (MODY2). There were however discrepancies in the first reports investigating Tmem27 function in pancreas β-cells that have not been solved. Overexpression of Tmem27 in mice showed that it either increases glucose-stimulated insulin exocytosis by binding snapin and therefore facilitating SNARE complex formation (Fukui et al, 2005), or by increasing β-cell mass (Akpinar et al, 2005).

Rarer mutations in HNF1β cause polycystic kidney disease additionally to the diabetes mellitus. This effect might be linked to the HNF1β-controlled expression of Tmem27 in the renal collecting duct where it is heavily expressed in the primary cilium. More specifically, it

localizes in the vesicles near the peri-basal body region and bound to β-actin-myosin II-A, SNARE and polycystin-2-polaris complexes (Zhang et al, 2007).

6.3 Nitric Oxide

Nitric oxide (NO), or endothelium-derived relaxing factor (EDRF) before it was molecularly identified, controls basal blood flow and blood pressure. It is also implicated in platlet aggregation, disaggregation and adhesion and in immune response by macrophages and neutrophils. Professors Robert Furchgott, Louis Ignarro and Ferid Murad received the Nobel price in Physiology or Medicine in 1998 for their discoveries concerning "nitric oxide as a signalling molecule in the cardiovascular system" (Raju, 2000).

NO is synthesised by NO synthases (NOS) by cleaving the guanidine nitrogen from the arginine precursor thereby co-releasing citrulline. Different isoforms of NOS are expressed in endothelial cells (eNOS / NOS III), in macrophages and neutrophils (inducible iNOS / NOS II) and in neurons (nNOS / NOS I).
In endothelial cells, eNOS activity is induced by acetylcholine, bradykinin, substance P, adenosine nucleotides, Ca^{2+} as well as shear stress. Intracellular Ca^{2+} release by these factors binds to cytosolic calmodulin (CaM) that then stimulates eNOS. NO being a small lipophilic gas with a short half life (in the range of seconds), it freely diffuses through the membrane to the neighbouring VSMCs. Activation of guanylyl cyclase by NO in VSMCs converts GTP to cGMP, which in turn allows cGMP-dependant kinase to phosphorylate various substrates. The myosin light chain kinase (MLCK) is activated and decreases interaction between actin and myosin, whereas inactivation of the Sarco/Endoplasmic reticulum Ca^{2+}-ATPase (SERCA) decreases cytoplasmic Ca^{2+} concentration altogether leading to VSMC relaxation (Figure 10) (Boron & Boulpaep, 2005).
Study of NO signalling with NOS competitive inhibitors like L-NMMA or L-NAME (N^G-monomethyl-L-arginine or N^G-nitro-L-arginine methyl ester, respectively) showed that there is a basal release of NO that regulates blood flow and blood pressure with a greater impact on the arterial than on the venous side of circulation (Vallance et al, 1989), as well as a greater impact on large arterioles (Griffith & Edwards, 1990). Importance of NO in systemic blood pressure control was later confirmed in the *eNOS* knock-out mouse model that had increased 24 h average blood pressure levels (+15 %), daily range (+44 %), and coefficient of variation (+26 %) (Van Vliet et al, 2003).

It has been shown that infusion of amino acids increases renal perfusion flow and glomerular filtration rate (Castellino et al, 1986). These effects may be mediated by NO, as they were inhibited by L-NMMA (Tolins & Raij, 1991). This was however not consistent with studies comparing the effect of glycine infusion to that of an amino acid infusion mix. While glycine alone also induced hyperfiltration and hyperemia that were attenuated and abolished, respectively, by L-NMMA pre-treatment, this was only modestly blunted and ineffective, respectively, for the amino acid mix (King et al, 1991).

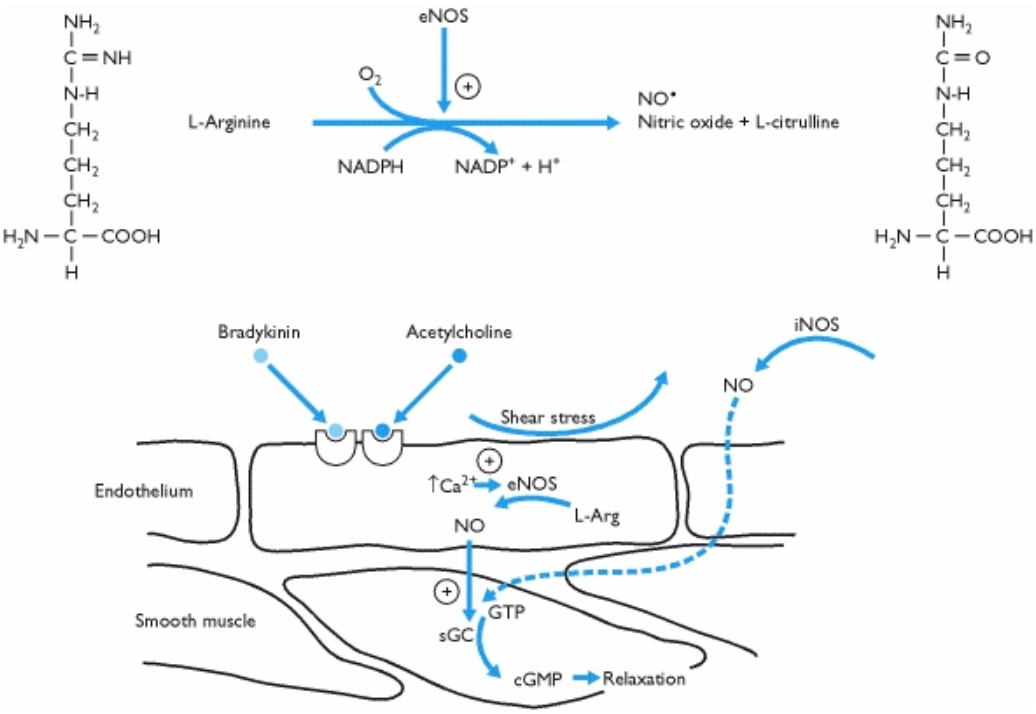

Figure 10. Nitric oxide (NO) synthesis cascade leading to smooth muscle cell relaxation. sGC: soluble guanylate cyclise (Nussey & Whitehead, 2001).

7. Original Research Article: Tissue-specific amino acid transporter partners ACE2 and Collectrin differentially interact with Hartnup mutations

This section contains an original research article that was accepted for publication in Gastroenterology and published online on October 30th 2008.

My contribution to this paper concerns the work performed *in vivo* in *ace2* and *coll* null mice which shows that B^0AT1 associates with tissue specific Tmem27 (Collectrin) and ACE2 in the kidney and in the small intestine, respectively.
The differential interaction of the associated proteins with wild type and mutations present in Hartnup disorder B^0AT1 was investigated in *X. laevis* oocytes by Dr. Simone M. R. Camargo with whom first authorship is shared.

The physiological impact of ACE2 loss on growth, metabolic parameters and amino acid levels was further investigated and reported in section 7.3.

Tissue-specific amino acid transporter partners ACE2 and Collectrin differentially interact with Hartnup mutations

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Short title: Hartnup disorder and ACE2

No conflict of interest exists

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Abbreviation List

AB, antibody

ACE2, angiotensin-converting enzyme 2

ace2 $^{-/-}$, *ace2* female null mouse

ace2 $^{-/y}$, *ace2* male null mouse

ace2 $^{+/+}$, *ace2* female wild type mouse

ace2 $^{+/y}$, *ace2* male wild type mouse

AP substrate, alkaline phosphatase substrate

B^0AT1, Broad range neutral amino acid transporter 1

BBMV, brush border membrane vesicles

coll $^{-/y}$, *collectrin* male null mouse

coll $^{+/y}$, *collectrin* male wild type mouse

C-term, Carboxyl-terminal

HRP substrate, horseradish peroxidase substrate

KO, knock-out mouse

MES, 2-(N-morpholino)-ethanesulfonic acid

MTSEA-biotin, 2-(aminoethyl)-methanethiosulfonate-biotin

NMDG, N-methyl-D-glucamine

N-term, Amino-Terminal

PCR, polymerase chain reaction

PVDF, Polyvinylidenfluorid

SARS, Severe acute respiratory syndrome

SARS-CoV, SARS coronavirus

SDS, sodium dodecyl sulfate

SLC6A19, solute carrier family 6, member 19

SNP, Single nucleotide polymorphism

TEVC, two electrode voltage clamp

V_h, holding voltage

WT, Wild type

Abstract

Background and aims: Hartnup amino acid transporter B^0AT1 (SLC6A19) is the major luminal sodium-dependent neutral amino acid transporter of small intestine and kidney proximal tubule. The expression of B^0AT1 in kidney was recently shown to depend on its association with Collectrin (Tmem27), a protein homologous to the membrane anchoring domain of angiotensin converting enzyme 2 (ACE2). Since Collectrin is almost absent from small intestine, we tested the hypothesis that it is ACE2 that interacts with B^0AT1 in enterocytes. Furthermore, since B^0AT1 expression depends on an associated protein, we tested the hypothesis that Hartnup-causing B^0AT1 mutations differentially impact on B^0AT1 interaction with intestinal and kidney accessory proteins. **Results**: Immunofluorescence, co-immunoprecipitation, and functional experiments using wild type and *ace2* null mice demonstrate that expression of B^0AT1 in small intestine critically depends on ACE2. Co-expressing new and previously identified Hartnup disorder-causing missense mutations of B^0AT1 with either Collectrin or ACE2 in *Xenopus laevis* oocytes shows that the high frequency D173N and the newly identified P265L mutant B^0AT1 transporters can still be activated by ACE2 but not Collectrin coexpression. In contrast, the human A69T and R240Q B^0AT1 mutants cannot be activated by either of the associated proteins although they function as wild type B^0AT1 when expressed alone. **Conclusion**: We thus demonstrate that ACE2 is necessary for the expression of the Hartnup transporter in intestine and suggest that the differential functional association of mutant B^0AT1 transporters with ACE2 and Collectrin in intestine and kidney, respectively, participates to the phenotypic heterogeneity of human Hartnup disorder.

Key words: Epithelial transport, enterocyte, amino acid transport, renin-angiotensin system, hereditary aminoaciduria, intestinal absorption

Introduction

Hartnup is an autosomal recessive disorder caused by mutations in the SLC6A19 gene that encodes the main epithelial neutral amino acid transporter B^0AT1[1, 2]. This disorder is characterized by neutral aminoaciduria due to impaired amino acid transport in kidney proximal tubule epithelial cells whereas the extent of intestinal transport impairment appears to be less consistent. Various other clinical symptoms such as pellagra-like rash, cerebellar ataxia or other neurological dysfunctions may be present in affected individuals, while other subjects remain, besides aminoaciduria, asymptomatic. Phenotypic variability might be partially explained by the differential impact of various mutations and the frequent compound heterozygosity.

Differential phenotypic effects of mutations could arise from differential interactions of B^0AT1 with tissue-specific modulatory and/or associated proteins. We and others have previously shown that B^0AT1 requires association with Collectrin for luminal surface expression in kidney proximal tubule, whereas Collectrin is nearly absent in small intestine, the other major site of B^0AT1 expression [3, 4]. Intriguingly, the closest homolog of Collectrin is the angiotensin-converting enzyme 2 (ACE2), which functions as a key carboxypeptidase enzyme in the renin angiotensin system. ACE2 is involved in heart and kidney pathologies [5] and has been identified as the SARS receptor *in vitro* [6] and *in vivo* [7]. Importantly, previous studies have reported ACE2 expression and SARS-CoV replication in human small intestine [8, 9].

We first investigated whether ACE2 associates with B^0AT1 in mouse small intestine. Immunofluorescence, co-immunoprecipitation and functional data presented here clearly demonstrate that ACE2 is the specific partner of B^0AT1 in small intestine and thus that the accessory protein of B^0AT1 is tissue specific.

We then tested the influence of Collectrin and ACE2 on the function of human B^0AT1 Hartnup-causing mutations in the *Xenopus laevis* oocyte expression system. Our results

suggest that specific defects in interaction with tissue specific accessory proteins may lead to differential defects in intestinal versus kidney (re)absorption of neutral amino acids and thereby participate to the variability of phenotypes observed in Hartnup subjects.

Methods

Animals

The *ace2* and *collectrin* wild-type (WT) and knock-out (KO) mice were housed in standard conditions and fed a standard diet. Generation of the KO mice was described elsewhere [3, 5]. Animals were either anaesthetised and perfused with a fixative solution for localization studies or euthanized to remove small intestine and kidneys. Scraped small intestine mucosa cells and total kidneys were frozen in liquid nitrogen for subsequent RNA extraction or brush border membrane vesicles (BBMV) preparation. All procedures for mice handling were according to the Swiss Animal Welfare laws and approved by the Kantonales Veterinäramt Zürich.

Organ fixation

Male mice were anesthetized with ketamine and xylazine (90 mg/kg body weight, Narketan 10, Vétoquinol, Lure, France) and Xylazine (10 mg/kg body weight, Xylazin, Streuli, Uznach, Switzerland) intraperitoneally and perfused through the left cardiac ventricle with phosphate-buffered saline (PBS, phosphate buffer, pH 7.4) followed by a buffered paraformaldehyde solution (4%, pH 7), as previously described [10]. Small intestine was then harvested, incubated overnight in paraformaldehyde solution, washed several times with PBS and stored in PBS-0.02% sodium azide at 4°C. Tissues were then mounted with Kryostat OCT (Medite, Nunningen, Switzerland), frozen in liquid propane and stored at – 80°C.

Immunofluorescence

Immunofluorescence was performed as previously described [11]. Primary antibodies were diluted (1:200) for rabbit affinity purified anti-mouse B^0AT1 [11], and (1:100) for affinity

purified goat anti-mouse ACE2 (R&D Systems, Minneapolis, USA). Secondary antibodies were diluted (1:500) for Alexa Fluor 488 donkey anti-goat IgG and Alexa Fluor 594 donkey anti-rabbit IgG (Molecular Probes, Invitrogen, Carlsbad, USA). Digital images were viewed by using a Nikon Eclipse TE300 epifluorescence microscope (Nikon Instruments Inc., Melville, USA) equipped with a DS-5M Standard CCD camera (Nikon) and acquired with NIS-Elements (Nikon).

Brush Border Membrane preparations

Brush Border Membrane Vesicles (BBMV) were prepared from small intestine mucosa cells and kidneys using the Mg^{2+} precipitation technique as described elsewhere [12].

Western blotting

Western Blotting was performed as previously described [11]. Primary antibodies were diluted to: 1:1000 goat affinity purified anti-mouse ACE2 (R&D), 1:1000 goat affinity purified anti-human ACE2 (R&D), 1:2000 or 1:1000 for rabbit affinity purified anti-mouse or anti-human B^0AT1, respectively [11] (Pineda, Berlin, Germany) and 1:10000 for mouse anti-mouse β-actin (Sigma, St Louis, USA). Secondary antibodies were diluted (1:5000) for ECL™ anti-rabbit or anti-goat IgG Horseradish Peroxidase linked fragment from donkey or mouse, respectively (Amersham Biosciences, Piscataway, USA and Pierce, Rockford, USA.) or anti-mouse IgG Alkaline Phosphatase Conjugate from mouse (Promega, Madison, USA). Antibody binding was detected with Immobilon Western Chemiluminescent HRP or AP substrate (Millipore, Billerica, USA) and chemiluminescence visualized with a DIANA III camera (Raytest, Dietikon, Switzerland).

Intestinal Ring Uptake

Uptake of radiolabelled L-isoleucine was performed as previously described [13] on ileum segments, with slight modifications. Briefly, everted ileum segments were incubated in bubbling (Oxycarbon) Krebs-Tris Buffer (pH 7.4) containing 1mM L-isoleucine (1 μCi ^{14}C-L-Ile/ml) for 5 min at 37°C. Ileum segments were dried at 55°C O/N on cellulose (Sartorius

AG, Goettingen, Germany) and weighed. Segments were then lysed in 0.75 N NaOH for 6 h, neutralised with 10N HCl and the radioactivity was determined by liquid scintillation. Na^+ was replaced by *N*-methyl-D-glucamine (NMDG) in the condition without Na^+. Amino acid transport was expressed relative to dry tissue weight.

Identification of new Hartnup mutations

After informed consent genomic DNA was obtained from whole blood of Hartnup patients as previously published [1]. Intronic primers to sequence exons and splice sites of each exon were chosen. Primer sequences are available on request. PCR products of each exon were separated by electrophoresis on agarose gels and specific bands removed for DNA isolation. This DNA was sequenced in both directions using a Beckman Coulter CEQ8000 (Beckman Coulter, Fullerton, USA) following the manufacturer's protocol. The absence of each recognized missense mutation was confirmed in 100 ethnically matched control alleles.

Isolation and subcloning of cDNAs and site-directed mutagenesis

Human (h) B^0AT1, ACE2 and Collectrin cDNAs were amplified from human kidney Marathon-Ready cDNA (Clontech, Mountain View, CA, USA) by PCR using a proofreading polymerase (Pfu, Promega, Madison, USA). The primers used are available upon request. The amplified ACE2 and Collectrin cDNAs were subcloned in pcDNA3; mouse (m) ACE2 and Collectrin in pcDNA 3.1 hygromycin (Invitrogen, Carlsbad, USA). The B^0AT1 was sub-cloned into a pBlueScript modified *Xenopus laevis* expression vector containing both the 5' and 3' ends of the β-globin gene (KSM) kindly provided by Dr. Leila Virkki. For expression in *Xenopus laevis* oocytes the plasmids were linearized and used as template for RNA synthesis (mMESSAGEmMACHINE™, Ambion, Austin, USA). The mutation in human B^0AT1 and ACE2 was performed using quick changes site-directed mutagenesis according to the manufacturer (Stratagene, La Jolla, USA). The mutation ACE2-R273Q was shown previously to be catalytically inactive but normally expressed [14]. The B^0AT1 mutations were done on the SNP V252I, which has been shown to behave like the wild type.

Homology model

The homology model of human B^0AT1 transport was based on the crystal structure of LeuT*Aa* (NP_214423) as a template using the I-TASSER server [15]. The pdb file was visualized using Pymol (DeLano Scientific LLC, Palo Alto, USA).

Transport studies in *Xenopus laevis* oocytes.

Expression studies and influx assays using radiolabeled amino acid tracer were performed in *X. laevis* oocytes after 5 to 9 days expression, as described previously [16]. Data is expressed in pmol / h / oocyte and values obtained for non-injected oocytes are subtracted.

Electrophysiology using two-microelectrode voltage clamp

The two-microelectrode voltage clamp (TEVC) technique was used for the recording of whole-cell currents from *X. laevis* oocytes. Recordings were performed at room temperature 5-9 days after injection with cRNA. Recordings were carried out as previously described [16] at a membrane holding potential (V_h) of −50 mV. To control for batch variation in transporter expression and measured current, the data from the selectivity experiment were normalized to $I_{L\text{-}Ile\ 10mM}$. Pooled data are shown as mean ± SEM where *n* represents the number of pooled cells. Experimental protocols were repeated at least twice. Nonlinear regression calculations were performed using GraphPad Prism™ Version 4.0 (GraphPad Inc., San Diego, USA).

Labelling of surface proteins in *Xenopus* oocytes

Surface labelling of oocytes expressing human B^0AT1 alone or co-expressed with mouse ACE2 or human Collectrin was performed using MTSEA-Biotin (Sigma-Aldrich, Switzerland) as previously described [17]. Samples were separated on a 10% SDS gel and immunoblotted with affinity purified rabbit anti- human B^0AT1 antibody (Pineda). Signal intensity was quantified with the AIDA Image Analyzer (Raytest).

Immunoprecipitations

Mouse brush border membrane proteins: BBMVs were incubated with serum anti-B^0AT1 polyclonal antibody [11] in EBC solution (20 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP40) at 4°C on a rotator. The immunocomplexes were coupled to Immobilized Protein A/G beads (Pierce) O/N at 4°C on a rotator. The beads were washed with NET-N solution (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5% NP40, 1 mM EDTA), and the immunoprecipitate loaded on a polyacrylamide gel. Western Blot was performed as described above with anti-mouse B^0AT1 antibody to check for B^0AT1 immunoprecipitation or anti-mouse ACE2 antibody to test ACE2 co-immunoprecipitation. To avoid background staining, Protein A/G coupled to Alkaline Phosphatase (AP) was used as a secondary antibody (Pierce).

Xenopus laevis oocytes: Immunopreciptation of human B^0AT1, B^0AT1 mutants and human ACE2 was performed as previously described [18]. Briefly oocytes were lysed in EBC buffer (as described for BBMV) and the supernatant was first incubated with serum anti-human B^0AT1 and subsequently with Immobilized Protein A/G beads (Pierce) O/N at 4°C on a rotator. Western Blot was performed as described above to test for human ACE2 co-immunoprecipitation.

Statistics

Data are presented as means ± standard error of the mean (SEM). Analyses were done by running the GraphPad Prism 4.0 software (GraphPad). Between-group comparisons were performed by Student's unpaired *t* test. Multiple comparisons within groups were performed by repeated-measures oneway ANOVA, followed by Tukey post test. Statistical significance was accepted at $P < 0.05$.

Results

ACE2 is the intestinal partner of the amino acid transporter B^0AT1

We first assessed the potential *in vivo* role of ACE2 as intestinal B^0AT1 associated protein by investigating the B^0AT1 expression in *ace*2 null mice [5]. Remarkably, B^0AT1 protein was completely absent in small intestine brush border membranes of mice lacking ACE2 (Figure 1A, B) whereas it was normally expressed in kidney (Figure 1B). This organ distribution of B^0AT1 in *ace2* null mice mirrors the situation observed in *collectrin* null mice, where B^0AT1 is absent in kidney and, as shown in this study, normally expressed in small intestine (Figure 1B). Furthermore, ACE2 was co-immunoprecipitated with B^0AT1 from intestinal brush border membranes of wild type mice, demonstrating *in vivo* interaction (Figure 1C).

The function of B^0AT1 was also shown to depend on ACE2 in mouse intestine. We observed that the lack of B^0AT1 protein expression in the intestine of *ace2*$^{-/y}$ mice abolished the Na^+-dependant portion of L-isoleucine transport measured in intestinal rings (Figure 1D). The total (in the presence of Na^+) but not the sodium-independent transport was dramatically reduced when compared to the wild type littermates. The equivalent effect was previously shown in kidney brush border membrane vesicles of *collectrin* $^{-/-}$ mice, where the transport was also reduced [3]. These data show that ACE2 is essential for expression and function of B^0AT1 in intestine whereas Collectrin controls B^0AT1 expression in the kidney.

ACE2 and Collectrin increase the function of human Hartnup transporter B^0AT1 *in vitro*

The tissue-specific associated proteins Collectrin and ACE2 expressed in *Xenopus laevis* oocytes mimicked their effect *in vivo* on B^0AT1 by stimulating its transport function. Expressed alone in oocytes human B^0AT1 induced a low amino acid transport rate as shown by us and others previously [1, 2]. We show here that its co-expression with mouse or human ACE2, or human Collectrin increases the transport function ~10-fold (Supplementary Figure 1A; see supplementary material online at www.gastrojournal.org). The mouse ortholog of Collectrin did not activate human B^0AT1, although it has been shown to be effective on

mouse B^0AT1 [3]. We have as yet no explanation for this differential impact of the collectrin orthologs in the *X. laevis* expression system. Different from Collectrin, ACE2 has one catalytic site. It is a carboxypeptidase, and converts Angiotensin (Ang) I and II to Ang (1-9) and (1-7), respectively [19, 20], functionally antagonizing its homolog ACE. We analyze the role of the catalytic activity of ACE2 in the functional interaction with B^0AT1 (Supplementary Figure 1B; see supplementary material online at www.gastrojournal.org). The catalytically dead mutant (ACE2 R273Q)[14] was as efficient as the wild type in enhancing the B^0AT1 function suggesting that it is not necessary for the interaction. This was expected since human Collectrin shares 48% identity with ACE2 at the level of its carboxyl-terminal membrane anchor region but entirely lacks the extracellular peptidase domain [21].

The amino acid selectivity, ion dependence and kinetic characteristics of human B^0AT1 co-expressed with ACE2 are, besides a much higher maximal transport rate, very similar to those previously published for human B^0AT1 expressed alone [1] (Supplementary Figure 2 A-E; see supplementary material online at www.gastrojournal.org).

Differential interaction of accessory proteins with B^0AT1 Hartnup mutations

To address the question whether Hartnup mutations impact on the interaction of B^0AT1 with its accessory proteins ACE2 and/or Collectrin, we expressed missense mutations alone or together with either accessory protein in *X. laevis* oocytes and analysed their transport function and surface expression. Beside five previously described missense mutations and one non-synonymous single nucleotide polymorphism (SNP), we characterized four new missense mutations identified in patients with neutral aminoaciduria characteristic of Hartnup disorder belonging to 4 different families (Table 1), (Supplementary Figure 3A-D; see supplementary material online at www.gastrojournal.org) [1, 2]. The non-synonymous SNP V252I essentially behaved as wild-type B^0AT1 when co-expressed with Collectrin and ACE2 (Figure 2A upper and middle panels). Interestingly, the observed two to three-fold increase in B^0AT1 surface

expression measured by surface biotinylation only partially explains the almost ten-fold increase in surface transport function induced by co-expression with Collectrin or ACE2 (Figure 2A lower panel and representative Western blot).

Surprisingly, Collectrin and ACE2 co-expression differentially impacted on the surface expression and function of the two B^0AT1 mutants D173N and P265L (Figure 2B). In both cases, co-expression with ACE2 increased the transport rate whereas co-expression with Collectrin either had the opposite effect or no effect (Figure 2B upper panel). The transport function of these mutants was shown to be Na^+-dependent as for wild-type B^0AT1 (Figure 3 upper and middle panels). Surface biotinylation experiments suggested that the differential function of these B^0AT1 mutants with the two associated proteins is due, in the case of D173N, to a difference in surface expression, whereas the surface expression appeared in the case of P265L to be similar with both associated protein (Figure 2B lower panel and representative Western blot). Finally, the mutants D173N and P265L co-precipitated with human ACE2, confirming that they interact with ACE2 (Figure 2D).

We next analyzed the two B^0AT1 mutations A69T and R240Q that when expressed alone display the same L-isoleucine transport function and ion dependence as wild type B^0AT1, whereas their transport function was not activated in the presence of either associated protein (Figure 2C upper and middle panels and Figure 3 lower panel). In the case of the newly described A69T, this was particularly surprising, since its surface expression was increased two to three-fold by co-expression with both accessory proteins (Figure 2C lower panel and representative Western blot) and its interaction with ACE2 confirmed by co-immunoprecipitation (Figure 2D). In contrast, the surface expression of R240Q did not appear to be increased by the presence of the accessory proteins (Figure 2C lower panel and representative Western blot), although the mutant transporter was co-immunoprecipitated with human ACE2 to some extent (Figure 2D).

The mutations R57C, L242P, and E501K have previously been tested by expression in *X. laevis* oocytes, and showed no transport capacity [1, 2]. Co-expression of these mutants with Collectrin and ACE2 did not change their impaired function and did not affect their cell surface expression (Figure 4). Similarly, the newly described mutations G93R and P579L lack function also in the presence of associated proteins. However, the surface expression of G93R was selectively increased by ACE2 (Figure 4 lower panel and representative Western blot).

Discussion

Recently we have reported that Collectrin (Tmem27) associates with and controls the expression of B^0AT1 in kidney proximal tubule [3, 4]. In this study, we demonstrated a novel and unexpected function of the important renin-angiotensin system enzyme ACE2 that associates with the luminal amino acid transporter B^0AT1 in small intestine and thereby controls its surface expression and function. B^0AT1 associates in a tissue specific manner with ACE2 in small intestine and with Collectrin in the kidney. Thus, *ace*2 and *collectrin* null mice can be used to study the role of the neutral amino acid transporter B^0AT1 in kidney and intestine independently.

The same effect was observed on human B^0AT1 ortholog expressed in the heterologous expression systems, *Xenopus laevis* oocytes. The co-expression of the two accessory proteins, ACE2 or Collectrin, increased the transport rate and the cell surface expression of the transporter. The analysis of the function and surface expression of B^0AT1-Hartnup causing mutations expressed alone or together with the two partner proteins allowed us to discriminate classes of mutants that might differentially impact on the phenotype of the patients.

The Hartnup-causing mutations in B^0AT1 are localized throughout the protein, as shown on the homology model depicted in Figure 5. This is also the case for the largest class of mutations mentioned here, the “dead” mutants, three of which were published earlier (R57C, L242P, E501K[1, 2]) and two of which are newly described here (G93R and P579L)

(Supplementary Figure 3 C and D; see supplementary material online at www.gastrojournal.org). These mutants do not display any measurable function which is consistent with the fact that, with one exception, they do not reach the plasma membrane, neither when expressed alone, nor upon co-expression with associated proteins.

The second class of mutants corresponds to those which expressed alone function as wild type B^0AT1 but are not stimulated by either associated protein. In the case R240Q, its surface expression is not increased upon co-expression with ACE2 or Collectrin, but co-immunoprecipitation demonstrates that its interaction with ACE2 is not abolished. Assuming a qualitative effect of the R240Q mutation on this interaction, our observation is nonetheless potentially compatible with the hypothesis recently put forward by the group of Broer, namely that the localization of this mutation at the surface of B^0AT1 might impact on the interaction with accessory proteins [22]. Further studies are necessary to clarify the mechanism by which this mutation, initially classified as a SNP, prevents the normal activation of B^0AT1 by the accessory proteins. In contrast, in the case of the other functional mutant of this class A69T, an increase in cell surface expression of this mutant B^0AT1 was demonstrated upon interaction with the associated proteins (Figure 2C and D). One possible explanation of why the increase in surface-expression did not lead to an increase in transport rate is that ACE2/A69T-B^0AT1 heterodimers reaching the cell surface remain inactive, meaning that the associated protein inhibits the function of A69T-B^0AT1. The fact that the residue mutated in A69T is part of a highly conserved motif (NGGG*A*F) shown to undergo conformational changes during the transport cycle is compatible with this hypothesis [23]. Additionally, that the co-expression of ACE2 or Collectrin apparently activates the transport rate of wild type B^0AT1 several-fold more than it increases its surface expression (~10-fold versus ~2.5-fold) also suggests that associated proteins may impact on transporter function, though normally by increasing the cycling rate.

The most intriguing class of mutants in regards to its potential phenotypic impact is represented by D173N and the newly described P265L. These mutants differentially interact with the two tissue-specific accessory proteins such that their function is stimulated only by the intestine-specific associated protein ACE2. Interestingly, the D173N allele is relatively frequent in unrelated Hartnup pedigrees and can also be observed in healthy Caucasians with a high heterozygote frequency (1:122 healthy individuals) [2, 24]. We know of no potential selective advantage for heterozygous carriers that explains the persistence and geographic spread of this mutant allele [25, 26]. The selective functional interaction of D173N with the intestinal accessory protein ACE2 might prevent deleterious effects due to a lack of amino acid absorption and thereby allowing the "survival" of this frequent allele. Although as yet no evidence of genotype-phenotype relationship performed in patients were reported, studies performed long before the identification of the Hartnup transporter have shown that intestinal amino acid absorption differs between Hartnup subjects [27, 28].

Taken together, our data demonstrate that the expression and function of the main epithelial amino acid transporter is modulated by tissue-specific accessory proteins. Moreover, the intriguing fact that these accessory proteins can interact differentially with Hartnup mutations and thereby differentially affect kidney and intestinal amino acid transport function suggests that Collectrin and ACE2 may also contribute significantly to the phenotypic heterogeneity among individuals with Hartnup disorder.

Table 1: Missense mutations in the SLC6A19/human B^0AT1 gene described in subjects with Hartnup disorder.

Protein	wt	mutation	Position from the atg	Position in the protein
R57C[1]	**C**gc	**T**gc	**169**	**1st TMS**
***A69T**	**G**cc	**A**cc	**205**	**2nd TMS**
***G93R**	**G**gg	**A**gg	**277**	**2nd TMS**
D173N[2]	**G**ac	**A**ac	**517**	**2nd EL**
R240Q[2]	**cGa**	**cAa**	**719**	**3th EL**
L242P[2]	c**T**g	c**C**g	**725**	**3th EL**
***P265L**	c**C**g	c**T**g	**794**	**6th TMS**
E501K[2]	**G**ag	**A**ag	**1501**	**10th TMS**
***P579L**	**C**cg	**T**cg	**1735**	**6th EL**

New mutations (*) identified in this study. The previously published mutations by Kleta et al. [1] and Seow et al. [2] are also listed. R240Q was initially described as SNP by Seow et al[2]. TMS = transmembrane segment, EL = extracellular loop.

Figure 1. ACE2 and B^0AT1 co-localization and functional interaction.

(**A**) Immunofluorescence analysis shows that B^0AT1 protein expression is lost in small intestine of *ace2* null mice. Small intestine sections from *ace2*$^{+/y}$ and *ace2*$^{-/y}$ were co-labelled with antibodies against ACE2 (green) and B^0AT1 (red), and additionally viewed by phase contrast (PC). **(B)** Western blot analysis shows that B^0AT1 is absent in small intestine but present in kidney brush border membranes of *ace2* null mice, whereas B^0AT1 is expressed in small intestine and absent from kidney brush border membranes in *coll* null mice. The loading of brush border membranes (2.5-20 μg) was tested using a β-actin antibody (βA). Note that male *ace2*$^{+/y}$ or *ace2*$^{-/y}$ and female *ace2*$^{+/+}$ or *ace2*$^{-/-}$ mice were used with similar results. **(C)** Co-immunoprecipitation shows interaction of ACE2 with B^0AT1 in mouse small intestine. Complexes were immunoprecipitated (IP) from small intestine brush border membrane vesicles (BBMVs) using anti-B^0AT1 antibody (AB) and analysed by Western Blot (WB) using anti-mouse ACE2 antibody. (**D**) Na^+-dependant uptake of L-isoleucine (L-Ile) is abolished in ileum segments from *ace2*$^{-/y}$ mice. The transport was measured in the presence (empty bar) and in the absence (solid bar) of sodium. Data points represent means values of 4 intestinal ring uptakes from independent experiments ± SEM, ***$p<0.001$, ns = not significant.

Figure 2. Differential function of Hartnup mutations co-expressed with Collectrin or ACE2 in *X. laevis* oocytes. Wild type and mutant human B^0AT1 are shown in this figure as three groups with different functional characteristics and labelled **A-C**. **(A)** The transport function and the surface expression of both wild type and non synonymous SNP V252I of B^0AT1 are increased by co-expression with human Collectrin or mouse ACE2. **(B)** The relatively low transport activity of B^0AT1 D173N and P265L mutants expressed alone is increased by co-expression with ACE2 but not with Collectrin. The surface expression of the D173N mutant is also significantly increased by co-expression with ACE2. **(C)** The wild type-like function of the A69T or R240Q mutants expressed alone is not increased in the

presence of Collectrin or ACE2 although; in the case of A69T its surface expression is increased. The upper panels of **A-C** show the transport function measured as radiolabelled L-isoleucine (L-Ile) uptake (means ± SEM of 13-31 oocytes from 3 independent experiments) and the middle panel the transport function as current measured by TEVC (9-11 oocytes from 3 batches). **P*<0.05, ****P*<0.001. The lower panels show the surface expression assayed by Western blot using surface-biotinylated proteins and a representative Western blot. The data correspond to the means ± SEM (n = 3-5 independent experiments) of the results normalized to the value obtained for B^0AT1 expressed alone. For the transport experiments the significance was evaluated using ANOVA with Tuckey post-test and for the surface biotinylation the comparison of means was done by unpaired *t* test. **(D)** B^0AT1 and the mutants D173N, P265L, R240Q and A69T interact with ACE2 in *X. laevis* oocytes. The transporters expressed alone or together with human ACE2 were immunoprecipitated (IP) from oocyte lysates using anti-human B^0AT1 antibody and complexes were analysed by Western Blot (WB) using anti-human ACE2 antibody. NI = non-injected.

Figure 3. The ion dependency of D173N, P265L, A69T and R240Q co-expressed with ACE2 or Collectrin is not altered: Transport is Na^+- but not Cl^--dependent. Wild type human B^0AT1 (WT), and the mutants D173N, P265L**,** A69T and R240Q were expressed alone or with human Collectrin or mouse ACE2. The ion dependence was tested by substituting Na^+ with NMDG (*n*-methyl-D-glucamine) and substituting Cl^- with gluconate. Function was assayed by influx of radiolabeled L-isoleucine (L-Ile). For each mutation, three independent experiments (15-24 oocytes) were pooled. Comparison of means was done by analysis of the variances (ANOVA) with Tuckey as post test. Data represent means ± SEM. * p<0.05, **p<0.01, ***p<0.001. ns, not significant.

Figure 4. The B^0AT1 mutants R57C, G93R, L242P, E501K and P579L exhibit no transport activity when expressed alone or co-expressed with Collectrin or ACE2. Function was assayed by influx of radiolabeled L-isoleucine (L-Ile) (upper panels) or TEVC (middle panels). For each mutant three independent experiments (13-31 oocytes for influx and 9-11 for TEVC) were pooled. Comparison of means was done by analysis of the variances (ANOVA) with Tuckey as post test. Data represent means ± SEM. ns, not significant. Cell surface expression of labelled proteins (lower panels) was analysed by Western blot. The bands were quantified and data normalized to the values obtained from oocytes expressing B^0AT1 proteins alone. For each mutation three to four independent experiments were pooled. Comparison of means was done by unpaired *t* test. Data represent means ± SEM. * $p<0.05$. ns, not significant.

Figure 5. Missense mutations in the SLC6A19 gene causing Hartnup disorder. The model of B^0AT1 is based on the Slc6 bacterial homologue from *Aquifex aeolicus* $LeuT_{Aa}$ [29] and the transmembrane segments are numbered. The localization of mutations causing a loss of surface expression (R57, G93, L242, E501, P579) are depicted in the left panel. The sites of mutations leading to a differential interaction with the two accessory proteins (D173 and P265) are depicted as black spheres on the middle panel, and the sites of the mutations preventing both accessory proteins of stimulating the transport function are depicted on the right panel (A69 and R240).

Appendix

Supplementary data

Supplementary data associated with this article can be found in the online version.

Acknowledgements

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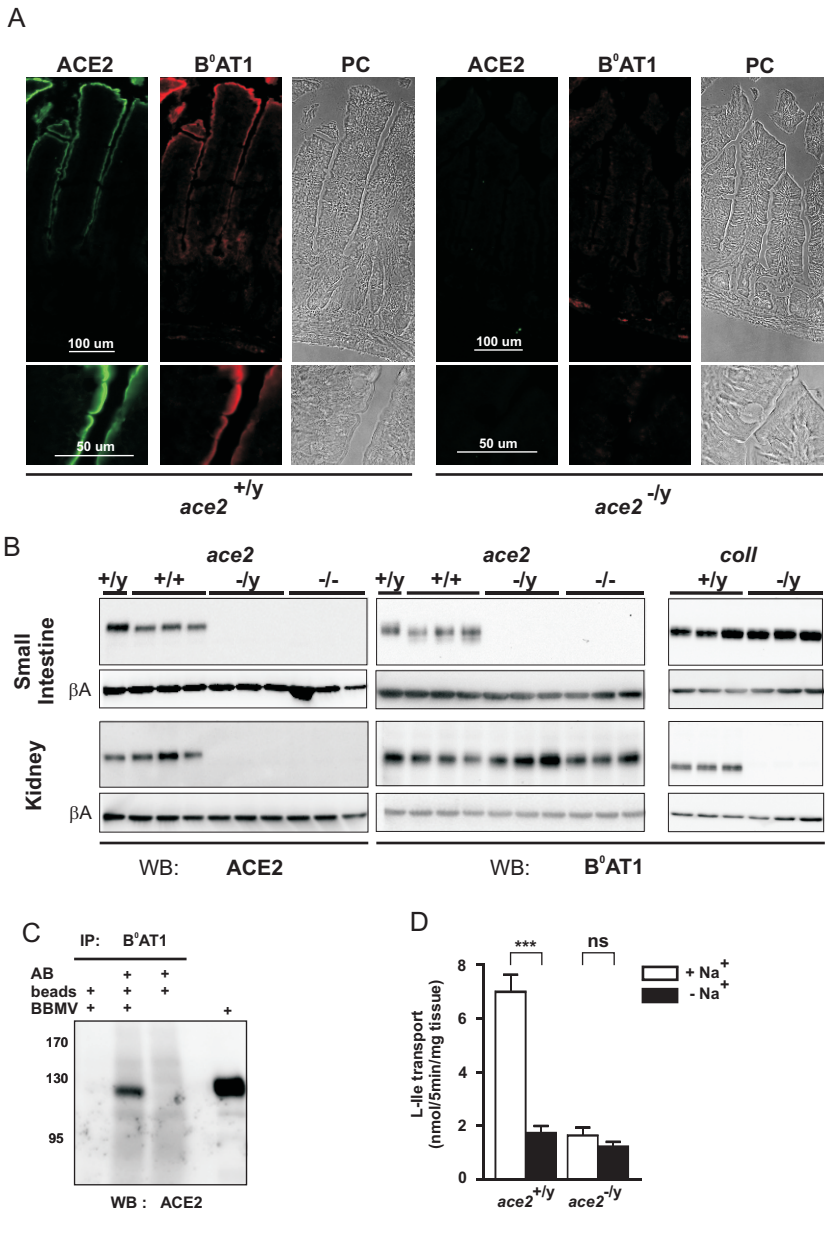
Figure 1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Kidney
βA
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WB: B⁰AT1
C
IP: B⁰AT1
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WB : ACE2
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Figure 2

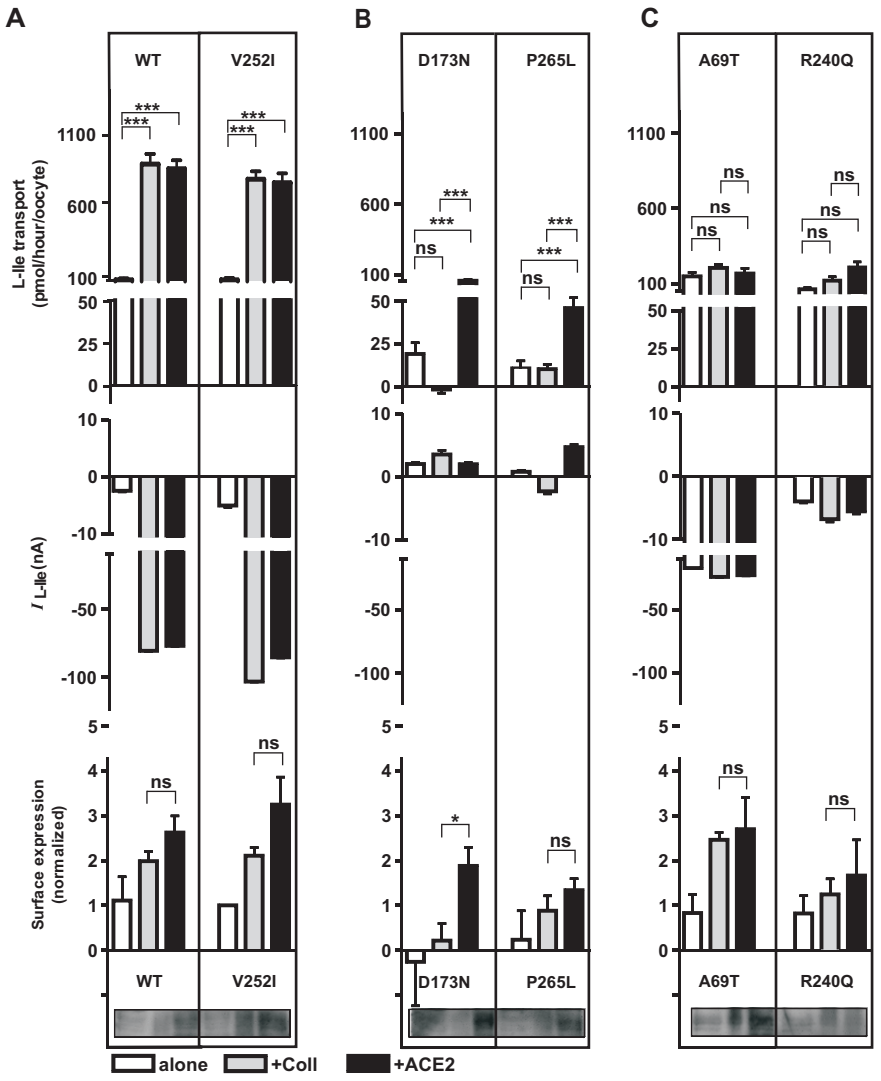
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Figure 3

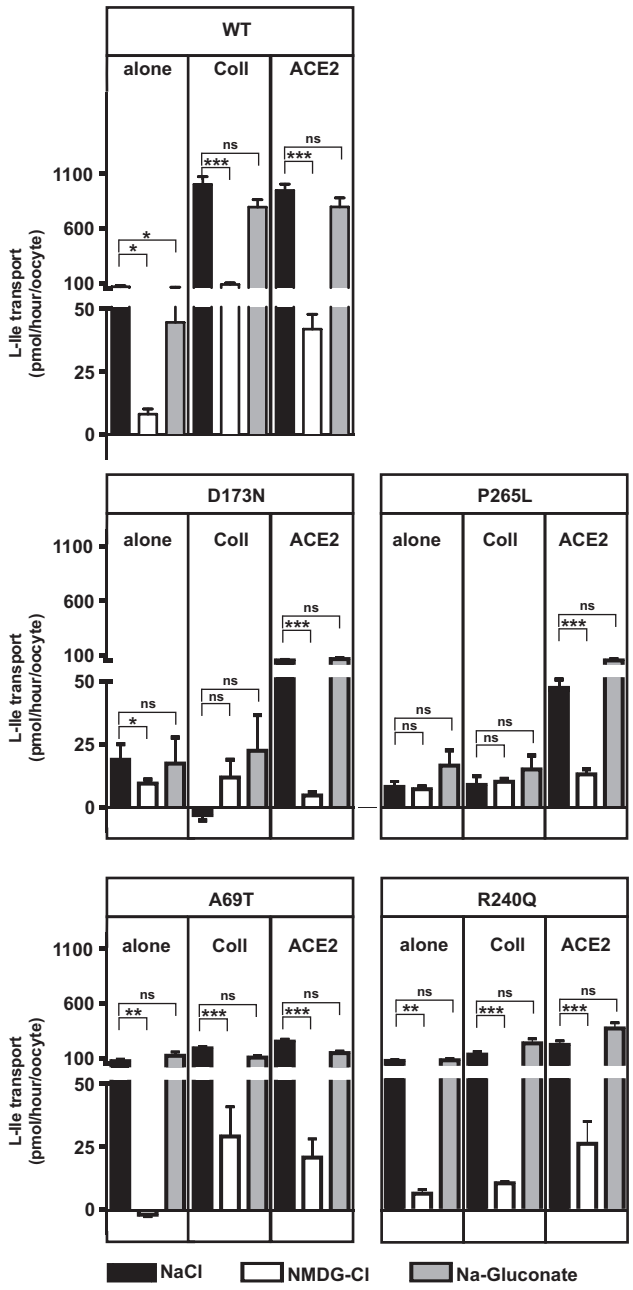
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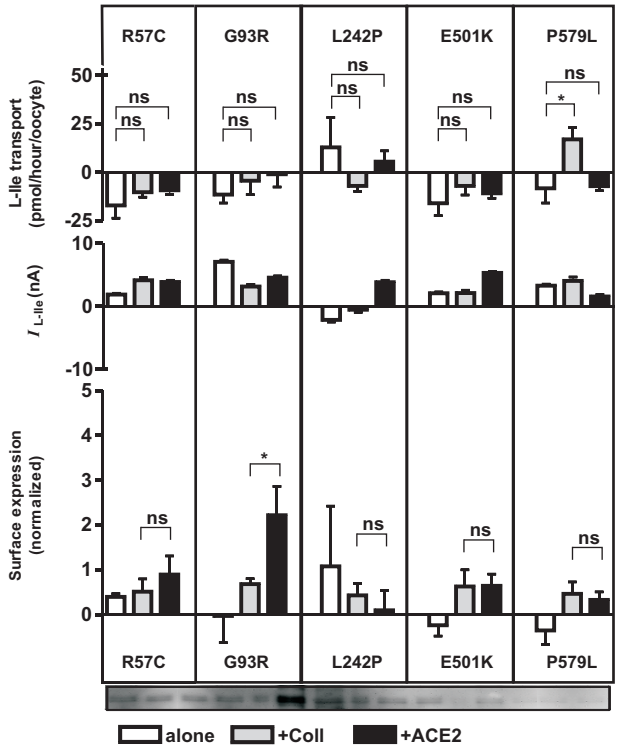
Figure 4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Surface expression (normalized)
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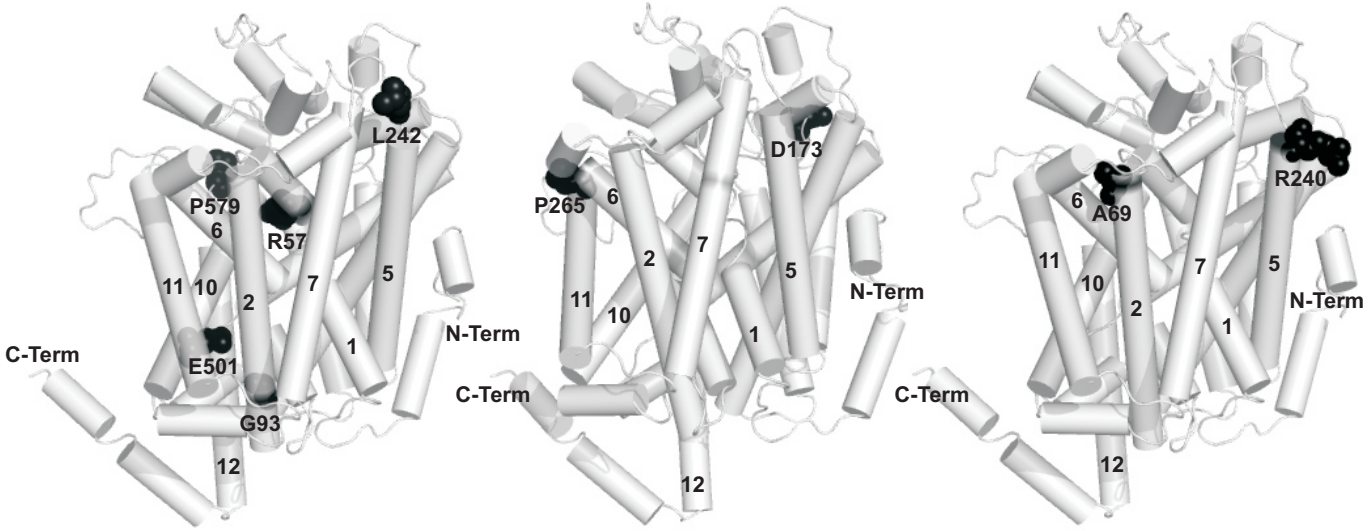
+ACE2

Figure 5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R57
11
10
2
7
5
1
N-Term
C-Term
E501
G93
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2
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11
10
5
N-Term
1
C-Term
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6
A69
R240
11
10
7
5
2
N-Term
1
C-Term
12

Tissue-specific amino acid transporter partners ACE2 and Collectrin differentially interact with Hartnup mutations

Simone M. R. Camargo*[1], Dustin Singer*[1], Victoria Makrides[1], Katja Huggel[1], Klaas M. Pos, Carsten A. Wagner[1], Keiji Kuba[2], Ursula Danilczyk[2], Flemming Skovby[4], Robert Kleta[3], Josef M. Penninger[2], and François Verrey[1] * These authors contributed equally to this work

Supplementary Figure 1. Function of human B^0AT1 co-expressed with orthologs of Collectrin and ACE2 in *X. laevis* oocytes

(**A**) Co-expression of ACE2 or human Collectrin increases B^0AT1 function in *X. laevis* oocytes. Tracer flux experiments (upper graph, n = 16-32) and two-electrode voltage clamp (TEVC, lower graph, n = 11) show that human B^0AT1 (hB^0AT1) co-expressed with human ACE2 (hACE2), mouse ACE2 (mACE2), or human Collectrin (hColl) transports L-isoleucine more efficiently than when expressed alone or in combination with mouse Collectrin (mColl). Data points represent means ± SEM. ***p<0.001. ns, no significant.

(**B**) Human B^0AT1 function is increased by co-expressed human ACE2 independent of its protease activity. The protease-dead human ACE2 mutant (ACE2 R273Q) stimulated the human B^0AT1-mediated L-isoleucine influx to the same extent as wild type human ACE2. Data points represent means ± SEM (n=15-16). ns, not significant.

Supplementary Figure 2. Functional characterization of the complex formed by human B^0AT1 and ACE2 in *X. laevis* oocytes. (**A**) The amino acid transport by human B^0AT1 co-expressed with mouse ACE2 in *X. laevis* oocytes is Na^+-, but not Cl^--dependent. The ion dependence was tested by substituting Na^+ by NMDG (*n*-methyl-D-glucamine) or Li^+, and Cl^- by gluconate in the superfusion solution used for TEVC. The values were normalized to the current obtained by superfusion of the oocytes with 10 mM L-isoleucine in solution containing Na^+ and Cl^- (I_{norm}). Comparison of means was done by analysis of the variances

(ANOVA) with Tuckey as post test. Each data point represents the mean ± SEM (n=16). *** p< 0.001.

(**B**) Amino acid transport by human B^0AT1 co-expressed with mouse ACE2 in *X. laevis* oocytes is maximal at neutral pH. The pH dependence of the transport was measured by TEVC in oocytes clamped at –50 mV and superfused with Na^+-solution buffered at pH 5.5, 6.5, 7.4 or 8.0. Values were normalized to the current obtained by superfusion of oocytes with 10 mM L-isoleucine in solution pH 7.4 (I_{norm}). Comparison of means was done by analysis of the variances (ANOVA) with Tuckey as post test. Each data point represents the mean ± SEM (n = 9). * $p < 0.05$, ** $p < 0.01$.

(**C-E**) Human B^0AT1 co-expressed with ACE2 transports a broad range of neutral amino acids with low affinity. (**C**), The substrate selectivity of human B^0AT1 co-expressed with mouse ACE2 was measured by TEVC in oocytes clamped at –50 mV and superfused with Na^+ solution containing 1 mM (open bars) or 10 mM (solid bars) of amino acids. The currents induced by the amino acids were normalized to the currents obtained in oocytes perfused with 10 mM L-isoleucine (I_{norm}). Data are shown as mean values ± SEM (n = 8-11) and the amino acids are indicated in single letter code. (**D, E**), The $K_{0.5}$ and V_{max} for L-isoleucine and Na^+ of human B^0AT1 co-expressed with mouse ACE2 was estimated using TEVC. For the Na^+ concentration dependence experiments the cells were first equilibrated with the indicated Na^+ concentration (0, 1, 5, 10, 30, 50, 70, and 100 mM) before the perfusate containing L-isoleucine (10 mM) was applied. For the L-isoleucine concentration dependence experiments, the cells were perfused with increasing concentrations of L-isoleucine (0.1, 0.3, 1, 3, and 10 mM). The continuous line is the nonlinear regression fit of the Michaelis-Menten equation to the data. Each data point represents the mean ± SEM (**C,** n = 26-27, for panel **D,** n = 10-22).

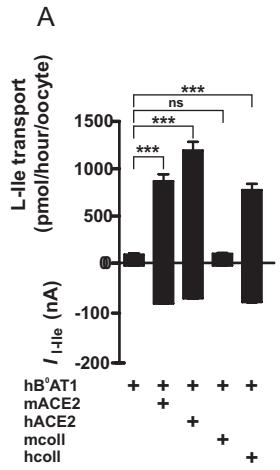
Supplementary Figure 3. Pedigrees of four families with new Hartnup disorder mutations. (**A**) Danish pedigree: two of three siblings were compound heterozygous for

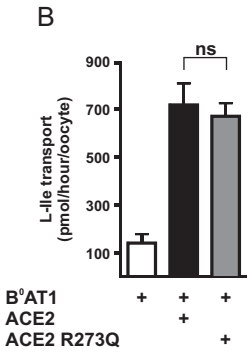
P265L and L242P. The symptoms observed were quite diverse between the two sisters. The younger one was diagnosed having Hartnup disorder in early childhood due to pellagra like skin rash and an atactic gait which improved upon niacin administration [1]. The older affected sibling never showed skin pathologies nor ataxia. (**B**) Another Danish pedigree, where the only child affected was a compound heterozygous for A69T and the original Hartnup mutation IVS8+2T>G. This patient was the first child of unrelated parents and was diagnosed at one year of age after a suspected seizure prompted a metabolic screening of urine. Plasma amino acids and electroencephalography were normal. The patient subsequently had normal growth and development without epilepsy, ataxia or pellagra. (**C**) The other Danish patient was a compound heterozygous for P579L and the original Hartnup mutation IVS8+2T>G. This patient was the second child of unrelated parents and was diagnosed at two years of age after onset of partial complex epilepsy with great difficulty to control seizures. The patient had normal plasma amino acids and never had ataxia or pellagra. (**D**) Another patient from a German family was diagnosed as homozygous for the new missense mutation G93R. This patient was diagnosed at six years of age during a migraine workup and also never had ataxia or pellagra. The affected subjects are depicted as filled symbols.

References

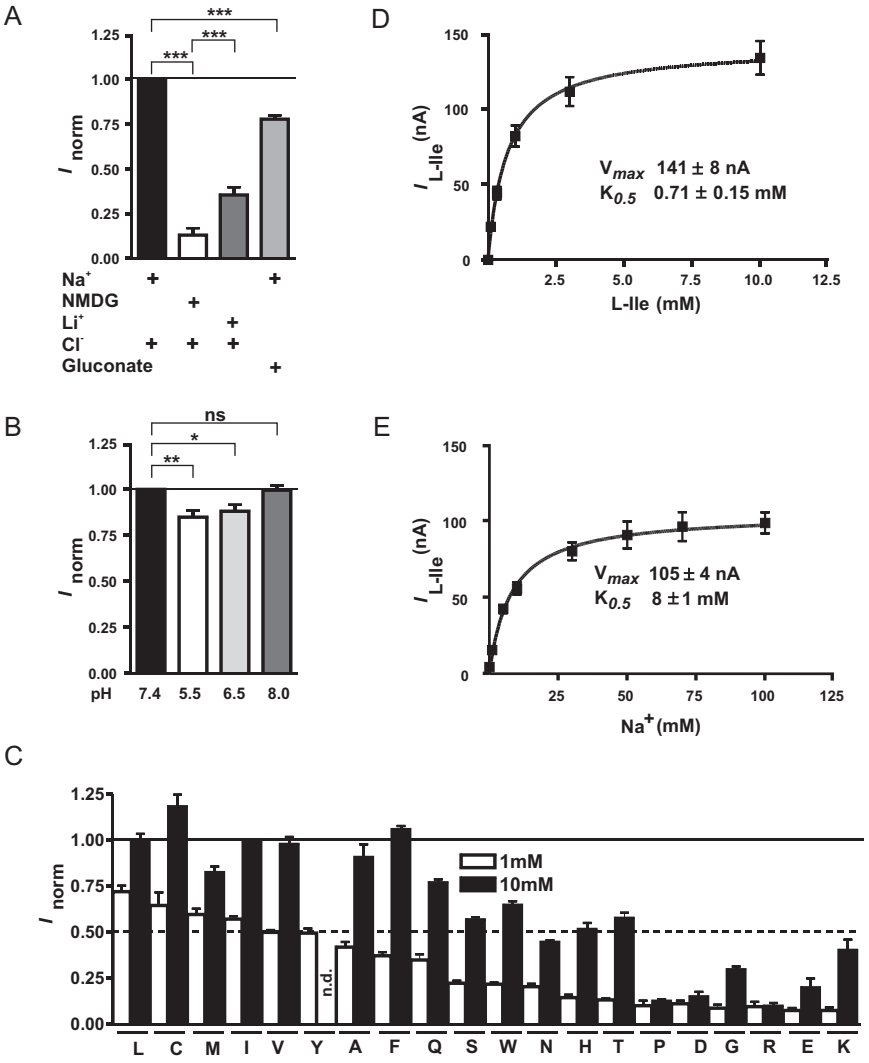
1. Nielsen EG, Vedso S, Zimmermann-Nielsen C. Hartnup disease in three siblings. Dan Med Bull 1966;13:155-61.

Supplementary Figure 1

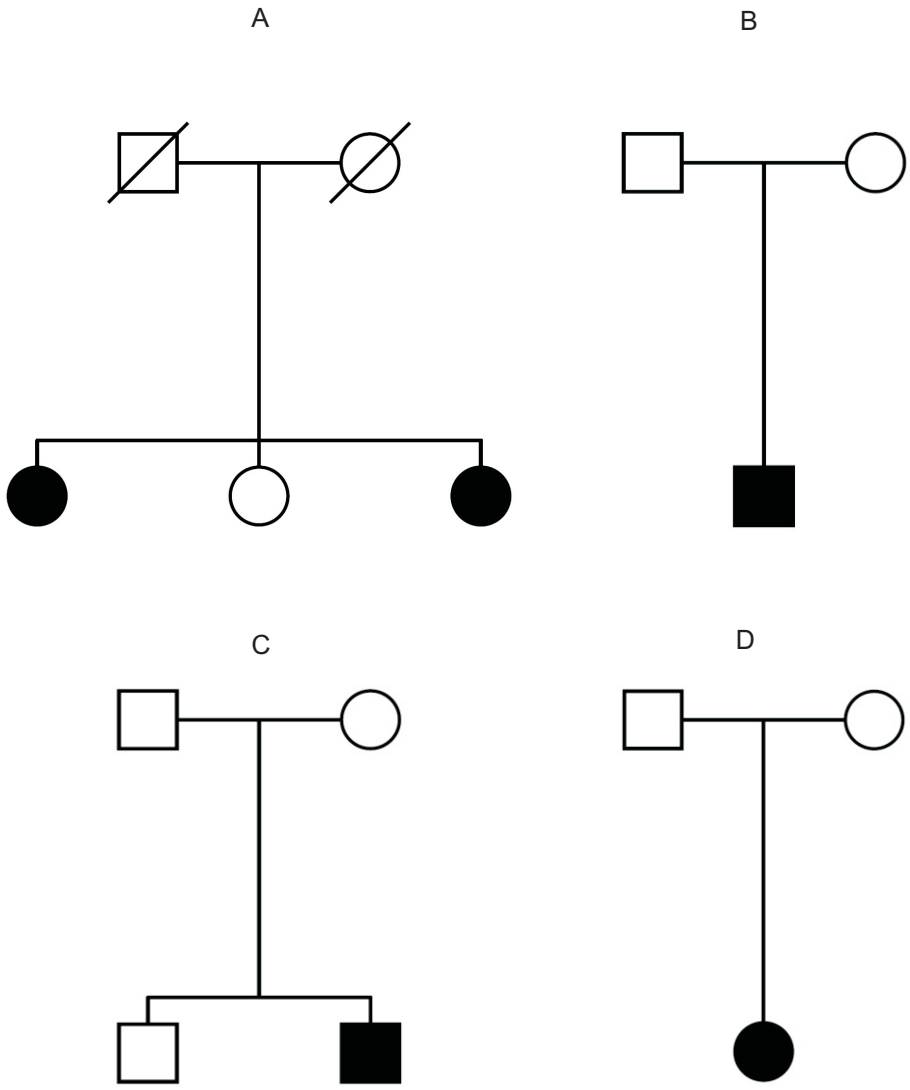




Supplementary Figure 2



Supplementary Figure 3



8. Manuscript: Orphan transporter SLC6A18 is renal neutral amino acid transporter B^0AT3

This section contains the above mentioned manuscript that has been submitted to the Journal of Biological Chemistry on October 3rd 2008. The editor sent it back on November 4th for additional experiments.

Dr. Simone M. R. Camargo significantly contributed to this research article by showing the amino acid selectivity, the Na^+ and Cl^- dependence and the kinetic parameters of B^0AT3 *in vitro* in *X. laevis* oocytes.

My contribution to the manuscript concerns the remaining work *in vivo.* Namely, the phenotypic characterisation of the *slc6a18* mouse model in metabolic cages and the blood pressure measurements using the telemetry implantation system, as well as the work with the *tmem27* and the *ace2* null mice to resolve the B^0AT3 associated protein issue *in vivo*.

Orphan transporter SLC6A18 is renal neutral amino acid transporter B^0AT3

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Running title : SLC6A18 is neutral amino acid transporter B^0AT3

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The orphan transporter Slc6a18 (XT2) is highly expressed at the luminal membrane of kidney proximal tubules and displays ~50% identity with Slc6a19 (B^0AT1) which is the main neutral amino acid transporter in both kidney and small intestine. As yet, the amino acid transport function of XT2 had only been experimentally supported by the urinary glycine loss observed in *xt2* null mice. We report here that in *Xenopus laevis* oocytes, co-expressed angiotensin-converting enzyme 2 (ACE2) associates with XT2 and leads to its cell-surface expression and reveals its function as an electroneutral Na^+- and Cl^--dependant neutral amino acid transporter. In contrast to its association with ACE2 observed in *Xenopus laevis* oocytes, our experiments with *ace2* and *collectrin* null mice demonstrate that *in vivo* it is Collectrin, a smaller homologue of ACE2, that is required for functional expression of XT2 in kidney. To assess the function of XT2 *in vivo*, we re-analysed its knock-out mouse model after more than 10 generations of backcrossing into C57BL/6 background. Next to the previously published glycinuria, we observed a urinary loss of several other, in particular beta-branched and small neutral amino acids. Using telemetry, we confirmed the previously described link of XT2 absence with hypertension, but only in physically restrained animals. Taken together, our data indicate that the formerly orphan transporter XT2 functions as a sodium and chloride dependent neutral amino acid transporter that we propose to rename B^0AT3.

The SLC6 family is composed of a variety of transporters that transfer small organic solutes such as neurotransmitters and amino acids across the plasma membrane. These substrates are co-transported with sodium and in many cases also with chloride. The substrates transported by most of the different family members have been identified and include neurotransmitters ((nor)epinephrine, dopamine, serotonin, GABA), osmolytes (taurine, creatinine) and amino acids. Despite this extensive characterisation, no data directly demonstrating the function of XT2 (Slc6a18) in an expression system have been reported. In particular, the expression of XT2 in *Xenopus laevis* oocytes and cultured mammalian cells failed to result in uptake of a variety of tested substrates (1,2).

However, XT2 exhibits approximately 50% identity to B^0AT1 (Slc6a19), the Na^+ cotransporter of neutral amino acids the defect of which causes

Hartnup disorder. The XT2 gene is arranged in tandem with that of B^0AT1 (Slc6a19) on chromosome 5 in human and chromosome 13 in mouse, and thus presumably arose by gene duplication. A cloning study identified, besides the A12 isoform that resembles most the other Slc6 family members, five shorter transcripts the physiological significance of which is not established (1). In a localization study performed using mouse tissues, we have shown that XT2 is mainly expressed in the kidney, where it localises to the brush border membrane of the late proximal tubule (S2, S3), in a complementary fashion to B^0AT1 that localises to the early proximal tubule (S1) (3).

A *xt2* null mouse model was generated and analyzed previously (2). The high amounts of glycine found in the urine of these mice supported the hypothesis that the orphan gene product XT2 functions as an amino acid transporter. Brush border membrane vesicles uptake additionally evidenced its function as a high affinity transport system of glycine. Surprisingly, the *xt2* null mice displayed a systolic blood pressure that was 15 - 20 mmHg higher than that of their wild-type littermates, a difference that was abolished upon glycine supplementation in drinking water. Such an impact of XT2 on blood pressure was however not confirmed in human, where a SNP within the SLC6A18 gene present in 46.7% of a general Japanese population corresponds to a nonsense mutation (Y319X) presumably leading to a loss of function, and is not associated with hypertension (4).

We have recently demonstrated that in the proximal kidney tubule, the expression of Slc6 B^0 cluster amino acid transporters requires their association with Collectrin (*Tmem27*, Coll), a membrane protein homologous to the membrane anchor domain of the renin angiotensin system enzyme ACE2 (5). Furthermore, we have shown that the enzyme ACE2, highly expressed in kidney and intestine, plays the role of associated protein in small intestine for luminal Slc6 amino acid transporters (S.M.R. Camargo and D. Singer et al., submitted)[†].

The first aim of the present study was therefore to retest the function of XT2 in the *X. laevis* expression system using co-expression of both the kidney and the small intestine associated proteins Coll and ACE2, and second, to retest the impact of the absence of XT2 in the *xt2* null mouse model after backcrossing it more than 10 times into C57BL/6 background. It appears, based on telemetric measurements, that XT2 is involved in blood pressure control only under stress conditions in the C57BL/6. We show in this study that the product of Slc6a18 called XT2 is a Na^+- and Cl^--dependent neutral amino acid transporter that in contrast to B^0AT1 is not electrogenic and displays a higher apparent affinity and a different preference for its substrates. Because of its broad transport selectivity for neutral amino acids we suggest to rename it B^0AT3.

Experimental Procedures

cRNA preparation of mouse XT2, Collectrin and ACE2 - The constructs of the A12 isoform of mouse XT2-KSM, Collectrin-pcDNA3.1Hygro and ACE2-pcDNA3 were linearized and used as template for RNA synthesis (mMESSAGE mMACHINE, Ambion, Austin, Texas, USA).

Transport studies in X. leavis oocytes- Expression studies and uptake measurements in *X. leavis* oocytes were performed as described previously (6). Ten minutes uptakes were performed with buffer containing 0.1 mM of the corresponding L-AA (2 uCi ^{14}C-L-AA / ml or 20 uCi ^{3}H-L-AA / ml). All experiments were done in buffer containing NaCl (100 mM) or NMDG-Cl (100 mM) for ion dependency experiments. Chloride was substituted by corresponding gluconate (Glc) salts. Kinetics experiments were performed with increasing concentrations of L-Ile (0.01, 0.03, 0.1, 0.3, and 1 mM) or L-Gly (0.01, 0.1, 0.3, 1, and 3 mM), in the presence of NaCl. Data is expressed in pmol / h / oocyte and values obtained for non-injected oocytes are subtracted. Multiple comparisons within groups were performed by repeated-measures one way ANOVA, followed by Tukey post test.

Animals - The *ace2* and *collectrin* wild-type and knock-out mice were housed in standard conditions and fed a standard diet. Generation of the knock-out mice was described elsewhere (5,7). All procedures for mice handling were according to the Swiss Animal Welfare laws and approved by the Kantonales Veterinäramt Zürich.

Metabolic Cages - Animals were adapted to metabolic cages (Tecniplast, Buguggiate, Italy) for 3 days before data collection, where they had free

access to standard mouse diet (18.5% crude protein, Kliba-Nafag, Kaiseraugst, Switzerland) and drinking water. After a first day of data collection in standard conditions, the diet was either switched to low protein (<0.5% crude protein, Kliba-Nafag) for two days or water access was removed for 24 h. Daily food / water intake, urine / feces output and body weights were measured. Urinary pH was measured using a pH microelectrode (691 pH-meter, Metrohm). Urinary creatinine was measured by the Jaffe method (8). Urinary electrolytes (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}) were measured by ion chromatography (Metrohm ion chromatograph, Herisau, Switzerland).

Amino acid analysis - Mouse blood was collected by decapitation and 1 µl of heparin-Na^+ 25000 I.E./5 ml (B. Braun, Melsungen, Germany) was added. Plasma was collected after centrifugation at 8000 rpm and 4°C. Ice-cold methanol deproteinization of the plasma was performed as described elsewhere (9). Hundred microliters of deproteinised sample was dried and resuspended in 45 µl of borate buffer. Samples were then derivatized using AccQ Tag (Waters, Milford, USA) and analyzed on an Acquity UPLC (Waters) according to the manufacturers instructions by the Functional Genomics Center Zurich (FGCZ) (10).

One microliter mouse urine collected over 24 h was diluted up to 100 µl by 50 mM HCl containing the internal standards norvaline and sarcosine in a concentration of 50 pmol/µl. The solutions were centrifuged, and transferred into new hydrolysing tube. One microliter was injected for pre-column derivatization with ortho-phthaldialdehyde (OPA) and analyzed on an Amino Quant amino acid analyzer (Agilent Technologies GmbH, Böblingen, Germany) at the Functional Genomics Center Zurich (FGCZ).

RNA and real time qRT-PCR - RNA was extracted from frozen kidneys and real time qRT-PCR was performed as previously described (3). Three samples per mouse and genotype were run and the abundance of the target mRNAs was calculated relative to Hypoxanthine Guanine Phosphoribosyltransferase (HPRT) or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference. Relative expression ratios were calculated as $R = 2^{(Ct(reference) - Ct(test))}$. Ct : cycle number at the threshold, test : tested mRNAs. Primers and probes were chosen as described elsewhere for B^0AT1 (3), XT3 (3), SIT1 (3), XT2 (3), ACE2 (11), Coll (5), GAPDH (3) and HPRT (12).

Protein Preparation and Western blot Analysis - Oocyte homogenization and protein preparation was done as described previously (13). For immunoprecipitation, the lysate equivalent to 8 oocytes was coupled to 25 µl of anti-XT2 serum (3) in EBC solution (20 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP40) for 8 h at 4°C on a rotator. The immunocomplexes were coupled to Immobilized Protein A/G beads (Pierce, Rockford, USA) O/N at 4°C on a rotator. The beads were washed 6 times with NET-N solution (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5% NP40, 1 mM EDTA), and the immunoprecipitate was eluted, denatured and reduced by heating at 95°C for 5 min in Laemmli Buffer. The equivalent of 2.4 oocytes was analysed by Western blotting. Brush Border Membrane Vesicles (BBMV) were prepared from whole mouse kidney using the Mg^{2+} precipitation technique as described elsewhere (14). Immunoblotting of the equivalent of 1 oocytes or 20 µg of BBMVs was performed as described elsewhere (3). Primary antibodies were diluted (1:2000) for rabbit Affinity Purified (AP) anti-B^0AT1 (3) / anti-XT2 (3) / anti-XT3 (3), (1:2000) for guinea-pig serum anti-Coll (antigen peptide : NH2-VQSAIRKNRNRINSAC-CONH2 as in (15), Pineda, Berlin, Germany), (1:1000) for AP goat anti-mACE2 (R&D Systems, Minneapolis, USA), and (1:10000) for mouse anti-mb-actin (Sigma, St Louis, USA). Secondary antibodies were diluted (1:5000) for ECL™ Anti-rabbit IgG Horseradish Peroxidase linked fragment from donkey (Amersham Biosciences, Piscataway, USA), (1:10000) for Anti-guinea pig IgG Horseradish Peroxidase (Sigma) and (1:10000) for Anti-mouse IgG Alkaline Phosphatase Conjugate (Promega). Antibody binding was detected with Immobilon Western Chemiluminescent HRP or AP substrate (Millipore, Billerica, USA). Chemiluminescence was detected with a DIANA III camera (Raytest, Dietikon, Switzerland). For quantification relative to b-actin, signal intensity was quantified with the AIDA Image Analyzer (Raytest).

Organ fixation - Mice were anesthetized with ketamine and xylazine (90 mg/kg body weight,

Narketan 10, Vétoquinol, Lure, France) and Xylazine (10 mg/kg body weight, Xylazin, Streuli, Uznach, Switzerland) intraperitoneally and perfused through the left cardiac ventricle with phosphate-buffered saline (PBS, 0.9% NaCl in 10 mM phosphate buffer, pH 7.4) followed by a buffered paraformaldehyde solution (4%, pH 7), as previously described (16). Kidneys were then harvested, incubated overnight in paraformaldehyde solution, washed several times with PBS and stored in PBS-0.02% sodium azide at 4°C. Tissues were then mounted with Kryostat OCT (Medite, Nunningen, Switzerland), frozen in liquid propane and stored at - 80°C.

Immunofluorescence - Immunofluorescence was performed as previously described (3). Primary antibodies were diluted (1:200) for rabbit Affinity Purified (AP) anti-mXT2 (3), (1:100) for AP goat anti-mACE2 (R&D), and (1:1000) for rabbit serum anti-mCollectrin (antigen peptide : NH2-CDPLDMKGGHINDGFLT-CONH2 as in (15), Pineda). Secondary antibodies were diluted (1:500) for Alexa Fluor 594 donkey anti-rabbit IgG and Alexa Fluor 488 donkey anti-goat IgG (Molecular Probes, Invitrogen, Carlsbad, USA). For actin staining, Texas Red - X phalloidin (Molecular Probes) was diluted (1:500). Digital images were viewed using a Nikon Eclipse TE300 epifluorescence microscope (Nikon Instruments Inc., Melville, USA) equipped with a DS-5M Standard CCD camera (Nikon) and acquired with NIS-Elements (Nikon).

Telemetry measurement of blood pressure - Eight weeks old mice were anaesthetised (see *Organ Fixation*) and implanted with PA-C10 transmitter (Data Sciences International (DSI), St. Paul, USA) through the carotid artery as previously described and following manufacturer information to place the pressure sensitive tip in the aortic arch (17,18). For analgesia purposes, 100 µl of bupivacain hydrochloride 0.25% (Bucain, DeltaSelect GmbH Pfullingen, Germany) was injected directly in the wound at the end of the procedure and carprofenum (5mg/kg, Rimadyl, Pfizer, New York, USA) was injected subcutaneously every 12 h for 2 days. Twenty-four hours continuous measurements were recorded after 2 weeks of recovery with Dataquest ART version 3.1 and RespiRate (DSI). Systolic, diastolic and mean blood pressure, heart frequency, activity and respiratory rate were analysed separately for the light vs. dark phases and activity=0 vs. activity>0. To mimic tail-cuff manometry stress, mice were restrained during 20 min in a perforated 50 ml Falcon tube, when the data was continuously measured.

RESULTS

Since XT2 has been shown to be associated in kidney proximal tubule with Coll (5), we tested the possibility that XT2 would be expressed functionally in *X. laevis* oocyte in the presence of this associated protein. XT2 was therefore co-expressed in *X. laevis* oocytes with Coll and/or its homologue ACE2 and the uptake of L-isoleucine was tested. Unexpectedly, uptake of L-isoleucine was observed only when XT2 was co-expressed with ACE2, whereas no transport was observed when XT2 was expressed alone or together with Coll (Fig. 1A). Furthermore, co-expression of ACE2 and Coll with XT2 did not further increase the transport rate compared to XT2 + ACE2.

The L-isoleucine transport induction upon ACE2 co-expression correlates with a slight increase of XT2 total protein in the oocytes (Fig. 1B). Furthermore, co-immunoprecipitation of ACE2 from oocyte membranes using XT2 antibody shows that ACE2 interacts with XT2 (Fig. 1C). These results suggest that ACE2 acts as a chaperone for XT2 and increases its stability in *X. laevis* oocytes. This co-expression renders the measurement of XT2 transport function possible in an expression system for the first time.

XT2 co-expression with ACE2 allowed the characterization of its transport selectivity, showing the transport of a broad range of neutral amino acids (A, M, V, I > G, S, L) (Fig. 2A). Concentration-dependence experiments with L-Ile and Gly revealed an apparent affinity of XT2 for these substrates ($K_{0.5}$ 0.21 mM and 0.53 mM, respectively) that is higher than that of B^0AT1 (Figs. 2B-C) (19). In contrast to B^0AT1, but similarly to other members of the Slc6 family such as the GABA transporter GAT1 (Slc6a1), the transport of L-Ile by XT2 is not only Na^+- but also Cl^--dependant as well as pH-independent (Figs. 2D-E). Because the transport activity of XT2 is not electrogenic, its function could not be tested using electrophysiological techniques (data not shown).

To assess the situation of the different partners *in vivo*, we first checked the localisation of XT2, Coll and ACE2 in mouse kidney. As previously shown, XT2 localises to the apical membrane in distal segments of the proximal tubule (S2,S3) and is not present in the initial S1 segment that begins at the glomerulus (3) (Fig. 3A). In contrast, ACE2 and Coll are both localised along the entire proximal tubule, but with opposed gradients increasing towards the distal and proximal tubular ends, respectively (Fig. 3A). This shows that the main site of ACE2 expression coincides with the localisation of XT2, suggesting that XT2 may interact with ACE2 in the proximal tubule, additionally to its known interaction with Coll (5).

To test the role of these two potential associated proteins on XT2 expression *in vivo*, we used *ace2* and *coll* null mice. At the mRNA level in kidney, XT2 expression was unchanged in both *coll (5)* and *ace2* null mice (Fig. 3B). However, Western blot analysis of kidney brush border membrane proteins showed that XT2 was absent in the $coll^{-/y}$ mice, in contrast to $ace2^{-/y}$ and wild-type mice (Figs. 3C-D). Immunofluorescence performed on kidney sections confirmed that XT2 is lacking in $coll^{-/y}$ mice proximal tubule, whereas it is normally expressed in $ace2^{-/y}$ as in wild-type control mice (Figs. 3E-F). These results confirm that the partner of XT2 in mouse kidney is indeed Coll and not ACE2, despite the fact that the expression gradient of Coll along the proximal tubule is opposed to that of both XT2 and ACE2 and that Coll unlike ACE2 does not allow XT2 expression in *X. laevis* oocytes.

To verify and examine the role of XT2 as an amino acid transporter more in detail, we re-analysed the *xt2* null mouse model (2) after more than 10 generations of backcrossing in C57BL/6 background. To evaluate whether the lack of XT2 induces compensatory changes, we examined the expression of the Slc6 family members B^0AT1 and XT3 and of the associated proteins ACE2 and Coll at the mRNA and protein levels in $xt2^{-/-}$ mice but observed no difference with wild-type littermate controls (Supplemental Figs. 1A-B).

To evaluate the impact of the loss of XT2 on the whole animal, we monitored food and water intake as well as urine and feces output in metabolic cages, but there were no differences in the measured parameters between the $xt2^{+/+}$ and $xt2^{-/-}$ mice (Supplemental Table 1). Analysis of plasma amino acids only suggested a slight but statistically not significant decrease of glycine (G) and L-arginine (R) in the $xt2^{-/-}$ mice as compared to the $xt2^{+/+}$ mice (Fig. 4A). A massive loss of glycine in the urine of the $xt2^{-/-}$ mice was confirmed and the loss of other neutral amino acids, notably L-glutamine (Q), was observed (Fig. 4B). However, calculating an approximated fractional excretion (based on an estimated glomerular filtration rate of 150 µl/min in females and 240 µl/min in males (20)) L-glutamine was only fractionally excreted to 2.20%, whereas glycine and L-methionine (M) were fractionally excreted up to 51.37% and 33.41% in the $xt2^{-/-}$ mice, respectively (Table 1). These results show that despite aminoaciduria, these mice do not exhibit any compensatory mechanisms, implying that the normal diet readily provides excessive amounts of amino acids. The urinary peak corresponding to L-arginine could unfortunately not be distinguished from that of taurine, preventing the evaluation of L-arginine loss in *xt2* null mice. Challenging *xt2* null animals with a low protein diet (<0.5% crude protein) didn't induce any additional difference in the metabolic cages parameters measured between *xt2* null and wild-type mice (Supplemental Table 1).

In the original study, *xt2* null mice displayed, compared to wild-type littermates, a surprising elevation of blood pressure as measured by tail cuff manometry. This difference was then abolished when drinking water was supplemented with glycine (0.1 g/ml) (2). The blood pressure of *xt2* null mice was now retested using mice backcrossed more than 10 times into C57BL/6 background and using telemetry (Datasciences). The measurements showed no differences in blood pressure between the $xt2^{-/-}$ and $xt2^{+/+}$ mice during the light phase when mice were not active (Fig. 5A). Similar results were obtained during the light and dark phases, whether the mice were active or not (data not shown). To test whether the previously measured increase in blood pressure was provoked by the stress of the tail cuff manometry method, telemetry measurements were repeated while physically restraining the mice. Submitting the animals to this stress provoked a higher mean blood pressure in $xt2^{-/-}$ mice than in wild-type litter-mates (130.8 ± 0.89 mmHg versus 125.3 ± 1.40 mmHg), and also a higher respiratory

rate (Fig. 5B). This suggests an increased stress-induced reaction possibly involving a dysregulation of the sympathetic nervous system in *xt2*$^{-/-}$ mice.

DISCUSSION

The first aim of this study was to directly characterise the function of the orphan transporter XT2 in the *X. laevis* oocytes expression system using its *in vivo* associated protein Coll. However, not Coll but only its homologue ACE2 supported the functional surface expression of XT2 in *X. laevis* oocytes such that its transport function of neutral amino acids expected from its homology with B^0AT1 could be measured. This raised the suggestion that ACE2 might interact with XT2 in mouse kidney in addition to Coll. However, our experiments with the *ace2* and *coll* null mice confirmed that the *in vivo* partner of XT2 is Coll and not ACE2. Indeed XT2 expression and localisation in the proximal kidney tubule was normal in *ace2* null mice and absent in *coll* null mice. This observation suggests the possibility that the rising implication of ACE2 as a key player of the renin-angiotensin system during evolution induced the appearance of Coll by duplication in order to take over its role in amino acid transport. It is however not clear why the restriction of functional XT2 association with ACE2 or Coll is just opposite in *X. laevis* oocytes as compared to the *in vivo* situation in kidney. Further experiments will be required to identify potential additional cell-specific players that account for this difference.

The functional expression of XT2 together with ACE2 in oocytes allowed the long sought characterisation of the function of this orphan transporter. Interestingly, its broad transport selectivity for neutral amino acids is clearly different from that of B^0AT1. Particularly, whereas beta-branched amino acids are very good substrates for XT2, as it is the case for B^0AT1, aromatic amino acids are apparently less efficiently transported. Importantly, L-Ala, L-Met, Gly, L-Ser and L-Cys are also efficiently transported.

In contrast to B^0AT1 that displays a Na^+-dependant electrogenic neutral amino acid transport, XT2 is shown here to display a transport mode that is not only Na^+, but also Cl^--dependant and non-electrogenic with an affinity for its preferred substrates that is higher than that of B^0AT1. In that sense, the axial arrangement of XT2 that follows B^0AT1 along the proximal tubule recalls the analogous sequential arrangement of low and high affinity glucose and peptide transporters SGLT2 and 1 and Pept1 and 2, respectively.

To correlate the measured *in vitro* function of XT2 with its *in vivo* function we re-analysed the *xt2* null mouse model. As the knock-out mice didn't display compensatory changes of other amino acid transporters of the SLC6 cluster, or of associated proteins, we hypothesise that the urinary phenotype of *xt2* null mice is essentially due to the absence of XT2. In accordance to the *in vitro* results we measured a broad aminoaciduria. Strikingly, there was not only a substantial loss of glycine (60-fold higher than in *xt2*$^{+/+}$) but also of L-glutamine (20-fold) and of all other neutral amino acids (Fig. 4). XT2 thus appears to be required for reabsorbing the leftover tubular amino acids that have not been reabsorbed in the early proximal kidney tubule segments by B^0AT1 or the amino acids that would eventually backleak into the late proximal tubule segments through the paracellular route (21). Additionally, XT2 might also be required for the reabsorption of neutral amino acids that the luminal heterodimeric cystine and cationic amino acids transporter $b^{0,+}AT$-rBAT releases in exchange for its uptake substrates in the tubular lumen. Thereby XT2 would interfere with L-arginine metabolism and nitric oxide (NO) synthesis (22). This latter hypothesis could be an explanation for the slightly decreased L-arginine plasma level and the tendency for elevated blood pressure observed in *xt2* null mice.

Interestingly, XT2 had originally been identified in rat kidney as a renal osmotic stress-induced transcript (ROSIT) (23). However, the experiments that we performed in mice could not verify such a regulation (E. Romeo and F. Verrey, unpublished results). This is either due to a species difference or to the less drastic conditions that we used which might not have been sufficient to trigger this regulation.

In their original description, *xt2* null mice displayed a significantly elevated blood pressure (2). This was however not observed in the present study after backcrossing these mice 10 times in a C57BL/6 background and using a telemetry system

for the blood pressure measurements. Previous reports have already evidenced discrepancies between tail cuff manometry and telemetric blood pressure measurements and also highlighted the importance of the genetic background. In particular, the original *xt2* null mouse had Sv129 genetic background, a strain that contains two renin genes and the renal blood flow of which was suggested to be more sensitive toward NO than that of C57BL/6 mice (24). We could however observe in the present study a stress-sensitive hypertension and a stress-induced increase in respiratory rate in *xt2* null mice, suggesting a possible impact of the lack of this transporter on regulatory functions of the sympathetic nervous system. Interestingly, plasma L-arginine of $xt2^{-/-}$ mice was slightly lower than in the $xt2^{+/+}$ mice and L-arginine is the precursor of nitric oxide that, when released by endothelial cells, leads to vasodilation. Slightly lower circulating and renal levels of L-arginine might therefore induce susceptibility to an increase in blood pressure (25).

The role of XT2 in human seems to be limited since SLC6A18 has not been found associated with iminoglycinuria (R. Kleta, personnal communication). Furthermore, a frequent SNP that leads to a stop codon in SLC6A18 has not been linked to increased blood pressure (4). However, it is not excluded that the individual variability in stress-induced blood pressure increase could be linked to the function of this renal neutral amino acid transporter.

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FOOTNOTES

† Camargo, S.M.R., Singer D., Makrides V., Katja Huggel K., Pos K.M., Wagner C.A., Kuba K., Danilczyk U., Skovby F., Kleta R. Penninger J.M., and Verrey F. **Tissue-specific amino acid transporter partners ACE2 and Collectrin differentially interact with Hartnup mutations.** Submitted.

ACE2 : Angiotensin-converting enzyme 2
Coll : Collectrin (Tmem27)
BBMV : Brush border membrane vesicles
HPRT : Hypoxanthine Guanine Phosphoribosyltransferase
GAPDH : Glyceraldehyde 3-phosphate dehydrogenase

FIGURE LEGENDS

Fig. 1. XT2 and ACE2 functional interaction *in vitro*. *A.* Co-expression of ACE2 but not of Coll supports the function of XT2 in *X. laevis* oocytes. *X. laevis* oocytes were injected with 10 ng of each cRNA and incubated for 6 days. Na^+-dependent uptake of L-Ile 0.1 mM (10 min) was assayed. The data represent 16-24 oocytes from 2-4 independent experiments. Bars indicate mean value ± SEM. *** P<0.001. *B.* Total membrane proteins of *X. laevis* oocytes were analysed by Western blot using anti-XT2 antibody. Kidney brush border membrane vesicles (BBMV) were used as control. *C.* Total membranes of *X. laevis* oocytes were used for immunoprecipitation with an anti-XT2 antibody and immunocomplexes were analysed by Western blot with anti-ACE2 antibody.

Fig. 2. Transport selectivity and kinetics of XT2 co-expressed with ACE2. *A.* Substrate selectivity of XT2 co-expressed with ACE2 in *X. laevis* oocytes. Na^+-Cl^--dependant uptake selectivity of L-AA (0.1 mM) was assayed for 10 min. Amino acids are abbreviated with the single letter code. *B, C.* Concentration dependence of the XT2-ACE2 mediated transport in oocytes. Curves corresponding to Michaelis-Menten kinetics were fitted to Na^+-Cl^--dependant uptake of L-Ile (*B*) and Gly (*C*). Data represent means of 18-39 oocytes ± SEM from 3-5 independent experiments. *D.* Transport induced by XT2 co-expressed with ACE2 is Na^+- and Cl^--dependant. Data are means of 13-16 oocytes ± SEM from two independent

experiments. *E*. Transport of L-Ile was not influenced by pH. The transport of L-Ile was assayed in the presence of Na^+ and Cl^- at different pH's. Bars and points indicate mean value from 8 oocytes ± SEM.

Fig. 3. Lack of XT2 in *coll*$^{-/y}$ but not in *ace2*$^{-/y}$ mouse kidney. *A*. Immunofluorescence of wild-type kidney co-labelled with XT2 and ACE2, and on a consecutive section co-labelled with Coll and Actin. G : glomerulus. *B.* Real time RT-PCR of XT2 and Coll on *ace2*$^{+/y}$ and *ace2*$^{-/y}$ total kidney RNA. Bars indicate mean value ± SEM, n=3 mice per genotype. *C, D.* Kidney brush border membrane vesicles (BBMV) (20 μg) from *coll*$^{+/y}$ and *coll*$^{-/y}$ mice (*C)* and *ace2*$^{+/y}$ and *ace2*$^{-/y}$ mice (*D)* were analysed by Western blot using antibodies against XT2, Coll (with a specific signal at 42 kDa, see supplemental data), ACE2, and β-actin (βa). Immunofluorescence of *coll*$^{-/y}$ kidney co-labelled with XT2 and ACE2 (*E),* and *ace2*$^{-/y}$ kidney labelled with XT2 and on a consecutive section with Coll (*F*). As shown also by Western blot in *C, D*, XT2 expression is lacking in kidney of *coll* null mice, whereas it is expressed in *ace2* null mice.

Fig. 4. The *xt2* null mice have increased levels of neutral amino acids in urine, and a slight but not significant decrease of plasma glycine (G) and L-arginine (R). Plasma (*A)* and urine (*B)* of *xt2*$^{+/+}$ and *xt2*$^{-/-}$ mice was deproteinized and analysed by HPLC. Bars indicate mean values ± SEM, n=7 (*A)* and n=6 (*B)* mice per genotype. *P<0.05, ** P<0.01, *** P<0.001. ND: not determined.

Fig. 5. The *xt2* null mice display a stress-induced increase of blood pressure and respiratory rate. The *xt2*$^{+/+}$ and *xt2*$^{-/-}$ mice were implanted with blood pressure telemetry sensors (DSI). Mean pressure, pulse pressure and respiratory rate (RCPM : Respiratory Cycle Per Minute) of *xt2*$^{+/+}$ and *xt2*$^{-/-}$ mice during the light period, when the mice were not active (*A*) or during a 20 min physical restrain stress (*B*). Bars indicate mean values ± SEM, n=7 mice per genotype. *P<0.05, ** P<0.01.

Table 1. Fractional urinary amino acid excretion

[1]Fractional excretion was calculated using an estimate of the glomerular filtration rate of 150 µl/min in females and 240 µl/min in males (20), the measured amino acid concentrations in plasma and urine and the volume of urine excreted over 24 h.

[2]ND: not determined.

	[1]Fractional excretion (%)		
	***xt2*$^{+/+}$**	***xt2*$^{-/-}$**	**fold change**
G	0.53	51.37	96.25
A	0.59	4.49	7.60
V	0.33	3.50	10.75
L	1.23	11.71	9.50
I	2.30	8.97	3.90
M	5.00	33.41	6.68
S	0.47	6.66	14.07
T	1.04	7.33	7.06
C	ND	ND	ND
P	0.33	0.59	1.79
N	2.91	6.76	2.33
Q	0.11	2.94	26.72
F	0.39	5.87	15.21
Y	0.34	2.14	6.38
W	1.97	4.24	2.16
K	1.58	2.98	1.88
R	[2]ND	ND	ND
H	1.74	9.66	5.56
D	2.62	4.12	1.57
E	11.46	16.03	1.40

Figure 1

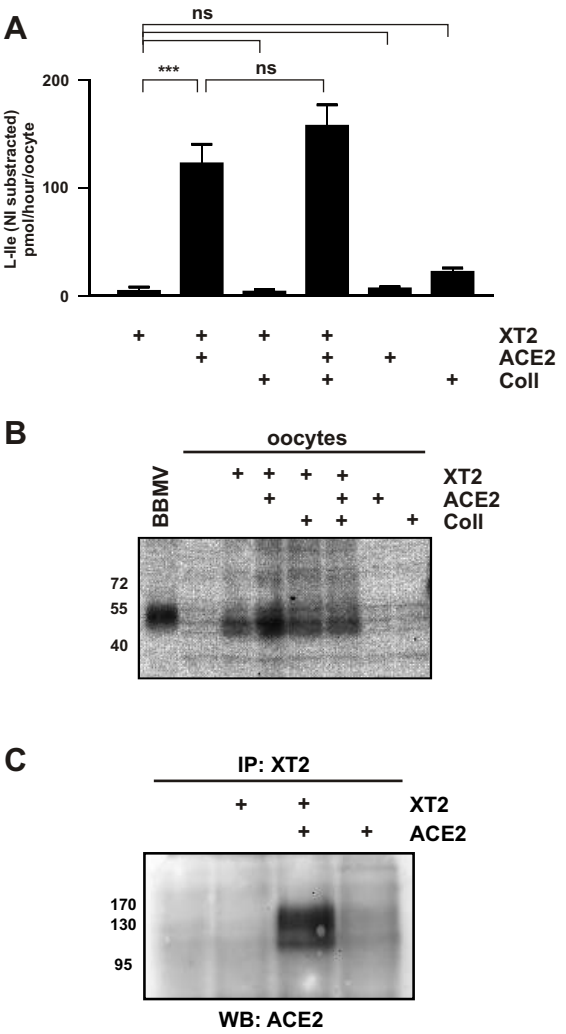
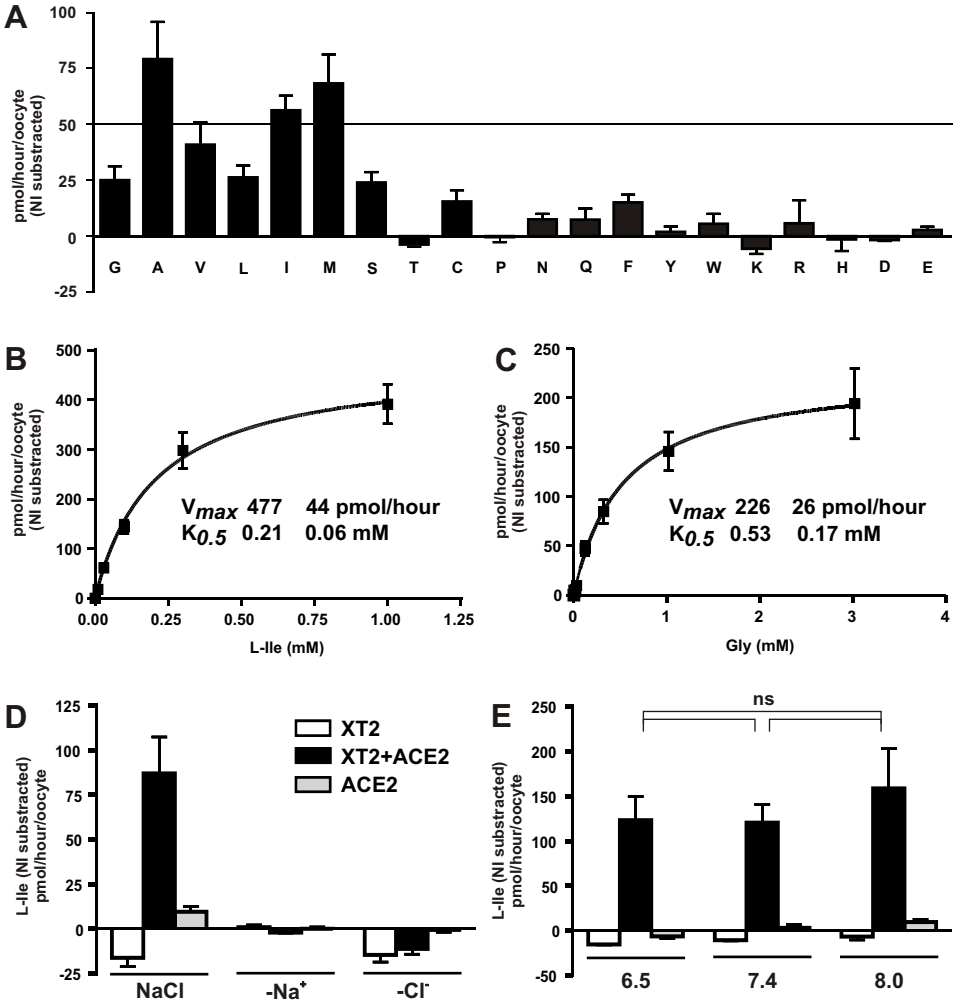

A
ns
***
ns
200
100
0
L-Ile (Nl substracted)
pmol/hour/oocyte
XT2
ACE2
Coll
B
oocytes
BBMV
XT2
ACE2
Coll
72
55
40
C
IP: XT2
XT2
ACE2
170
130
95
WB: ACE2

Figure 2


A
pmol/hour/oocyte (NI substracted)
100
75
50
25
0
-25
G A V L I M S T C P N Q F Y W K R H D E
B
pmol/hour/oocyte (NI substracted)
500
400
300
200
100
0
Vmax 477 44 pmol/hour
K0.5 0.21 0.06 mM
0.00 0.25 0.50 0.75 1.00 1.25
L-Ile (mM)
C
pmol/hour/oocyte (NI substracted)
250
200
150
100
50
0
Vmax 226 26 pmol/hour
K0.5 0.53 0.17 mM
0 1 2 3 4
Gly (mM)
D
L-Ile (NI substracted) pmol/hour/oocyte
125
100
75
50
25
0
-25
XT2
XT2+ACE2
ACE2
NaCl
-Na+
-Cl-
E
L-Ile (NI substracted) pmol/hour/oocyte
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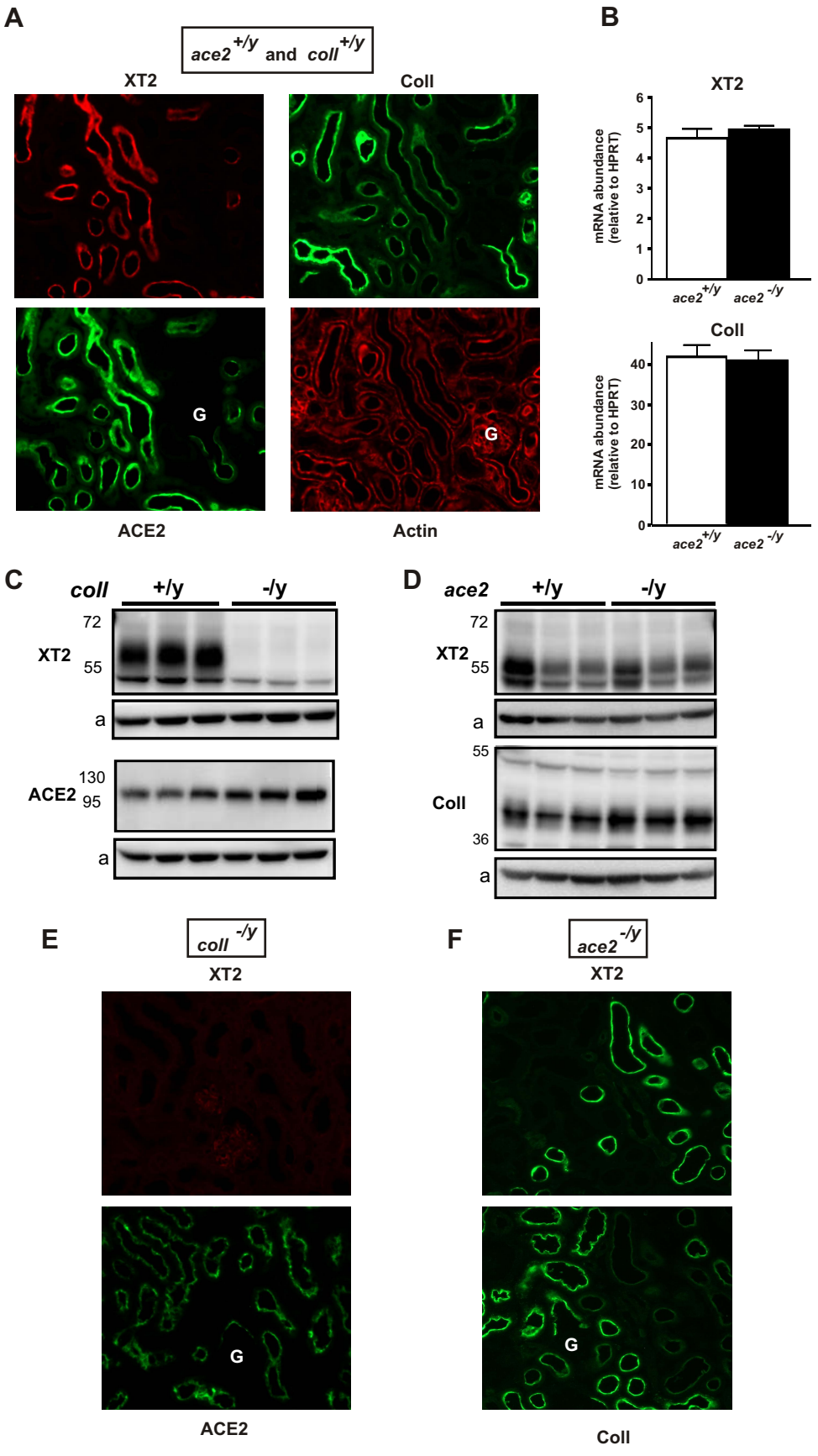
200
150
100
50
0
-50
ns
6.5
7.4
8.0

Figure 3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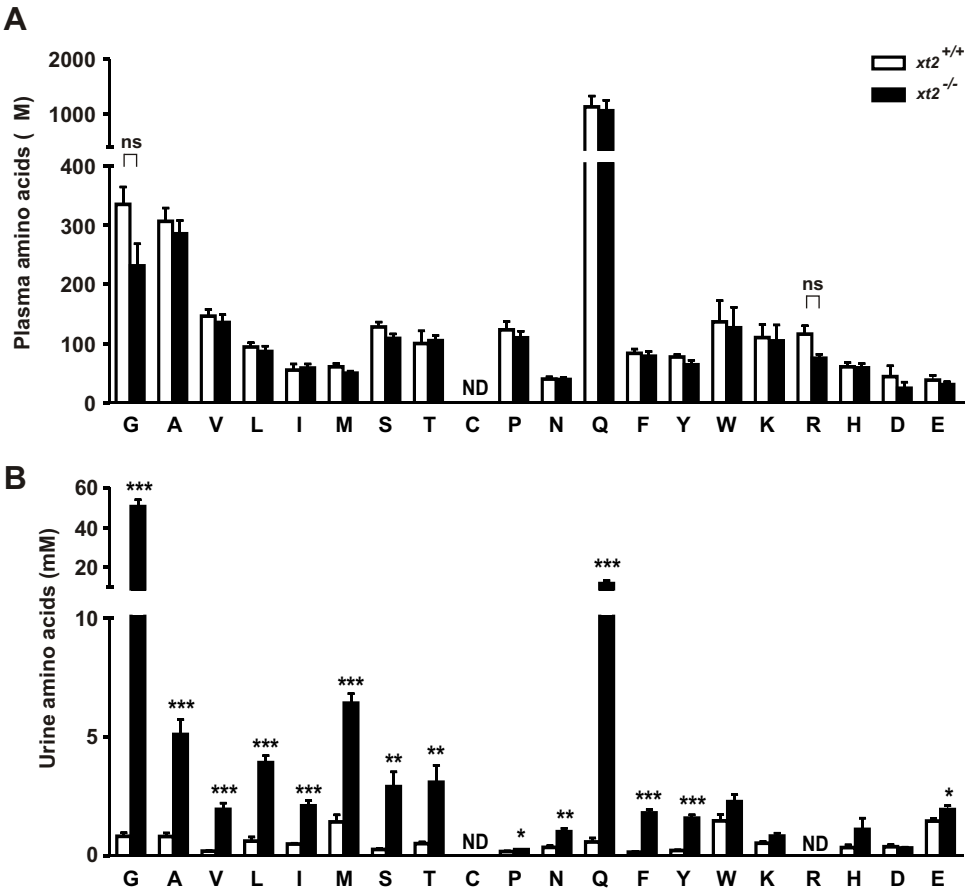
ace2+/y and coll+/y
XT2
Coll
G
G
ACE2
Actin
B
XT2
mRNA abundance (relative to HPRT)
6
5
4
3
2
1
0
ace2+/y
ace2-/y
Coll
mRNA abundance (relative to HPRT)
40
30
20
10
0
ace2+/y
ace2-/y
C
coll
+/y
-/y
72
XT2
55
a
130
ACE2
95
a
D
ace2
+/y
-/y
72
XT2
55
a
55
Coll
36
a
E
coll-/y
XT2
G
ACE2
F
ace2-/y
XT2
G
Coll

Figure 4


A
Plasma amino acids (M)
2000
1000
400
300
200
100
0
ns
ns
ND
xt2+/+
xt2-/-
G A V L I M S T C P N Q F Y W K R H D E
B
Urine amino acids (mM)
60
40
20
10
5
0
***
***
***
***
***
***
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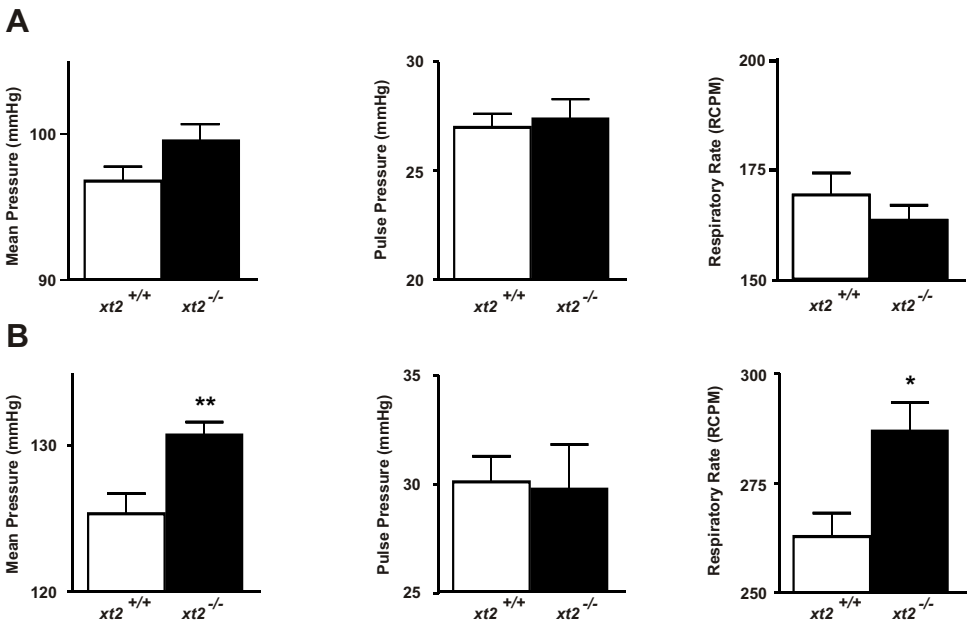
*
ND
G A V L I M S T C P N Q F Y W K R H D E

Figure 5


A
Mean Pressure (mmHg)
100
90
xt2 +/+
xt2 -/-
Pulse Pressure (mmHg)
30
25
20
xt2 +/+
xt2 -/-
Respiratory Rate (RCPM)
200
175
150
xt2 +/+
xt2 -/-
B
Mean Pressure (mmHg)
**
130
120
xt2 +/+
xt2 -/-
Pulse Pressure (mmHg)
35
30
25
xt2 +/+
xt2 -/-
Respiratory Rate (RCPM)
300
*
275
250
xt2 +/+
xt2 -/-

Orphan transporter SLC6A18 is renal neutral amino acid transporter B^0AT3 Supplemental Data

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* These authors contributed equally to this work

Supplemental Table 1. Summary of metabolic cage and urine data from male and female mice.
Male and female mice were fed a normal protein diet (*A.* 18.5 %, 2 d), a low protein diet (*B.* <0.5 %, 2 d) or were deprived of water (*C.* 24 h) *A.* Males n=9, females n=8, total n=17. *B.* Males n=4, females n=5, total n=9. *C.* Males n=5, females n=3, total n=8. Data indicate means ± SEM. Urinary excretion was normalized to creatinine (crea). BW: body weight. ND: not determined.

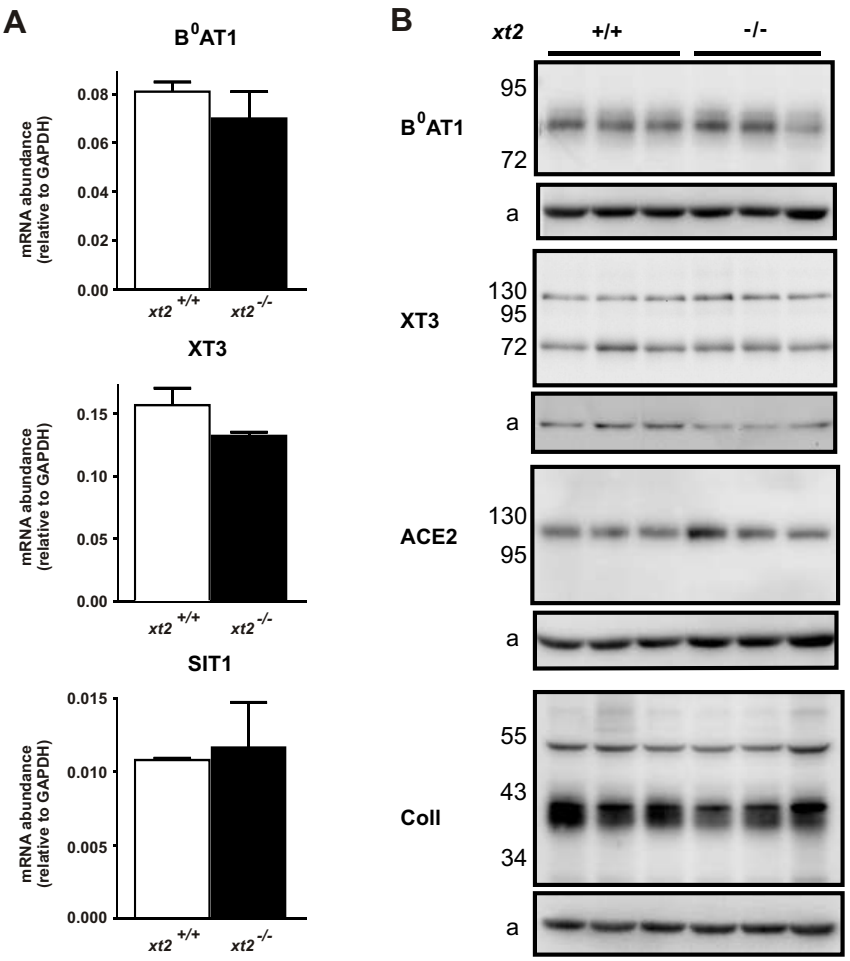
	[A]Normal protein diet (2 d)		[B]Low protein diet (2 d)		[C]Water deprivation (24 h)	
	$xt2^{+/+}$	$xt2^{-/-}$	$xt2^{+/+}$	$xt2^{-/-}$	$xt2^{+/+}$	$xt2^{-/-}$
Males body weight (g)	25.28±0.39	25.62±0.51	22.98±0.50	23.73±0.33	23.36±0.19	23.08±0.75
Females body weight (g)	21.65±0.43	21.65±0.39	20.28±0.09	20.44±0.35	19.17±0.82	18.93±0.65
Males and females :						
Food (g/g BW)	0.155±0.0075	0.158±0.008	0.142±0.005	0.147±0.003	0.110±0.010	0.113±0.005
Water (ml/g BW)	0.24±0.02	0.22±0.01	0.45±0.05	0.39±0.05	0	0
Feces (g/g BW)	0.056±0.004	0.053±0.005	0.018±0.004	0.018±0.002	0.0322±0.003	0.0310±0.002
Urine (ml/g BW)	0.031±0.005	0.039±0.005	0.200±0.026	0.162±0.023	0.012±0.003	0.018±0.003
Creatinine (mM)	5.44±0.42	5.19±0.34	1.40±0.17	1.39±0.17	5.82±0.61	6.15±0.43
Osmolality (mOsm/kg)	3266±209	3079±14	457.3±44.2	509.8±47.0	4680±356	4540±377
pH	6.23±0.07	6.28±0.12	6.22±0.14	6.27±0.06	ND	ND
Na^+/crea	33.08±4.08	38.44±4.71	81.93±5.20	122.9±21.4	44.11±7.38	41.91±8.60
K^+/crea	112.9±13.0	122.2±14.8	58.02±5.19	78.99±11.29	127.8±2.285	111.1±11.66
Ca^{2+}/crea	0.97±0.24	1.08±0.22	3.15±1.193	2.26±0.25	0.36±0.07	0.28±0.07
Mg^{2+}/crea	10.25 ± 1.11	11.44 ± 1.56	8.10 ± 0.73	10.19 ± 1.56	8.76 ± 0.50	9.52 ± 0.73
Cl^-/crea	58.78 ± 1.90	60.15 ± 2.55	83.54 ± 2.72	100.4 ± 12.12	135.9 ± 21.12	154.5 ± 26.59
PO_4^{2-}/crea	9.03 ± 0.64	10.05 ± 1.23	28.62 ± 1.61	30.56 ± 1.58	31.50 ± 5.24	27.18 ± 4.34
SO_4^{2-}/crea	14.34 ± 0.87	14.13 ± 0.74	4.82 ± 0.48	4.83 ± 0.38	24.82 ± 2.71	28.62 ± 5.06

Supplemental Fig. 1. Absence of compensatory regulation of other renal SLC6 amino acid transporters or associated proteins in *xt2* null mice. *A.* Real time RT-PCR of B^0AT1, XT3 (Slc6a20a) and SIT1 (Slc6a20b) on $ace2^{+/y}$ and $ace2^{-/y}$ total kidney RNA. Bars indicate mean value ± SEM, n=2 mice per

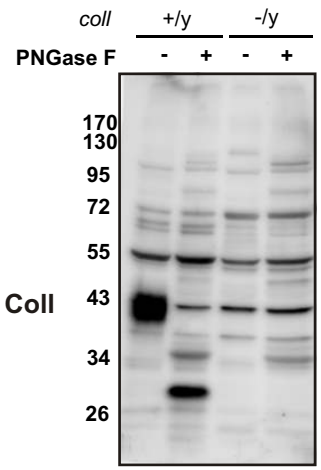
genotype. *B.* Western blot analysis on kidney BBMV (20 μg) from $xt2^{+/+}$ and $xt2^{-/-}$ mice using antibodies against B^0AT1, XT3, ACE2, Coll, and β-actin (βa).

Supplemental Fig. 2. Specificity of the guinea-pig serum anti-Coll was tested on $coll^{+/y}$ and $coll^{-/y}$ BBMVs with and without deglycosylation (PNGase F). A specific signal is detected around 40 kDa. Hydrolysis of Asn-oligosaccharides was carried out using endo-beta-N-acetylglucosaminidase F enzyme (PNGase F) (New England Biolabs, USA).

Supplemental Figure 1



Supplemental Figure 2



9. Physiological impact of ACE2 loss on amino acid transport

9.1. Rationale

As presented in section 7.2, *ace2* null mice have a defect in protein expression of the neutral amino acid transporter B^0AT1 in the brush border of the small intestine. The following section evaluates the impact of such a defect on mouse development. The phenotype reported in this section is presumably only partly due to the defect of intestinal B^0AT1 amino acid transporter. For instance, the control of the proline transporter SIT1 (Slc6a20) by ACE2 is not yet clear and requires further investigation. Furthermore, possible cardiac and hypertensive phenotypes and increased Ang II levels might additionally play a role on mouse development, although their effects have been described in older mice (6 months).

9.2. Material and Methods

Animals

The *ace2* wild-type and knock-out mice were housed in standard conditions and fed a standard diet. Generation of the knock-out mice was described elsewhere (Crackower et al, 2002). All procedures for mice handling were according to the Swiss Animal Welfare laws and approved by the Kantonales Veterinäramt Zürich.

Metabolic cages

Animals were adapted to metabolic cages (Tecniplast, Buguggiate, Italy) for 3 days before data collection, where they had free access to standard mouse diet (18.5% crude protein, Kliba-Nafag, Kaiseraugst, Switzerland) and drinking water. Daily food / water intake, urine / feces output and body weights were measured. Urinary pH was measured using a pH microelectrode (691 pH-meter, Metrohm). Urinary creatinine was measured by the Jaffe method (Seaton & Ali, 1984). Urinary and plasma urea were measured using the diacetyl monoxime method (Wybenga et al, 1971). Urinary electrolytes (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}) were measured by ion chromatography (Metrohm ion chromatograph, Herisau, Switzerland).

Mouse blood was collected by decapitation and 1 ul of heparin-Na^+ 25000 I.E./5 ml (B. Braun, Melsungen, Germany) was added. Plasma was collected after centrifugation at 6000

g and 4°C. Ice-cold methanol deproteinization of the plasma was performed as described elsewhere (Suresh Babu et al, 2002). Hundred microliters of deproteinised sample was dried and resuspended in 45 ul of borate buffer. Samples were then derivatized using AccQ Tag (Waters, Milford, USA) and analyzed on an Acquity UPLC (Waters) according to the manufacturers instructions by the Functional Genomics Center Zurich (FGCZ) (Cohen & De Antonis, 1994).

One microliter mouse urine collected over 24 h was diluted up to 100 ul by 50 mM HCl containing the internal standards norvaline and sarcosine in a concentration of 50 pmol/ul. The solutions were centrifuged, and transferred into new hydrolysing tube. One microliter was injected for pre-column derivatization with ortho-phthaldialdehyde (OPA) and analyzed on an Amino Quant amino acid analyzer (Agilent Technologies GmbH, Böblingen, Germany) at the Functional Genomics Center Zurich (FGCZ).

Growth Curve

Pups from *ace2*$^{+/-}$ x *ace2*$^{+/y}$ breedings weights were recorded starting one day after birth. Identification was possible by marking the pups' body using a Securiline alcohol-resistant lab marker (Precision Dynamics Corporation, San Fernando, USA). The tail was similarly marked when fur appeared on the body. At day 21, pups were weaned from the mother, separated by sex and biopsies were sampled for genotyping.

Statistics

Data are presented as means ± standard error of the mean (SEM). Analyses were done by running the GraphPad Prism 4.0 software (GraphPad). Between-group comparisons were performed by Student's unpaired t-test. For comparisons of weight differences between *ace2*$^{+/y}$ and *ace2*$^{-/y}$ during the growth experiments, the average weight was calculated for the genotype for each litter and analysed by a paired t-test. Statistical significance was accepted at $P < 0.05$.

9.3. Results

9.3.1. Lack of ACE2 affects plasma amino acids levels

We first measured plasma amino acid levels of *ace2* null mice to evaluate how amino acid homeostasis was affected by loss of intestinal B^0AT1. Decrease of plasma amino acid levels did not cover the whole range of B^0AT1 neutral amino acids substrates but was specific for glycine (G) and L-tryptophan (W). Surprisingly, this was accompanied by an increase of L-lysine (K) (Figure 1).

To estimate amino acid transporter function in the kidney of *ace2* null mice, we calculated the expected fractional excretion rates of amino acids in the urine using an estimate of glomerular filtration rate. As suggested by the normal expression levels of B^0AT1 in kidney brush border, fractional excretion of amino acids in the urine were not significantly modified (Table 1).

We have previously shown that the Na^+-dependant transport of L-isoleucine is absent in isolated intestine rings of $ace2^{-/y}$ mice (Camargo et al, in press). Since L-isoleucine is an essential amino acid, its transport in a di-/tri-peptide form through peptide transporter PepT1 seems to sufficiently compensate for lack of B^0AT1. The mRNA expression levels of PepT1 were however not increased (data not shown).

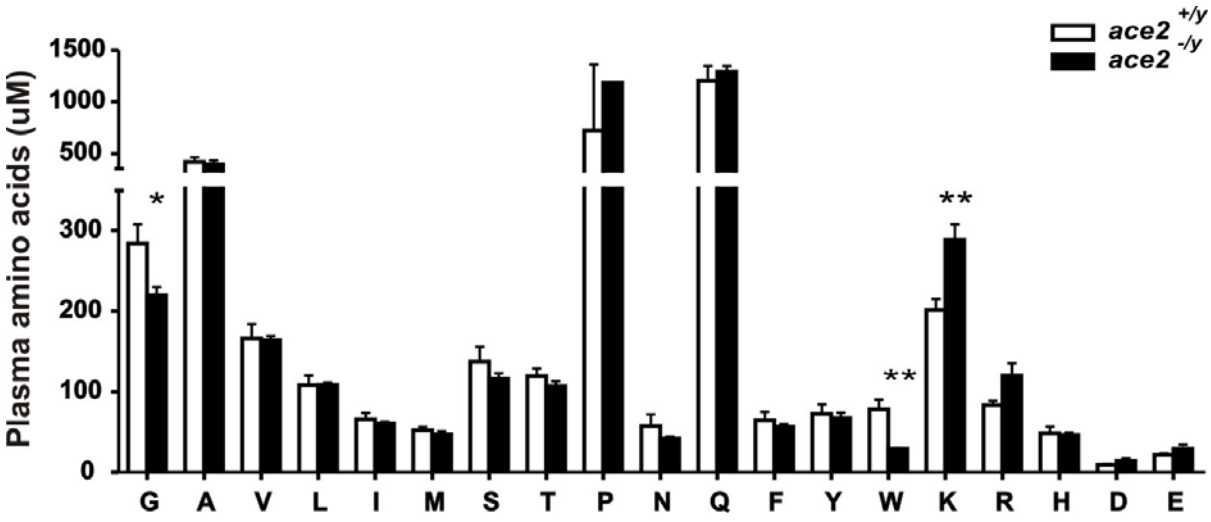


Figure 1. The *ace2* null male and female mice have decreased plasma levels of L-tryptophan (W) and glycine (G). Plasma of $ace2^{+/y}$ n=11 and $ace2^{-/y}$ n=6 mice was deproteinized and analysed by HPLC. Mean ± SEM. * P<0.5, ** P<0.01

	[1]Fractional excretion (%)		
	[2]*ace2*$^{+/y}$	*ace2*$^{-/y}$	fold change
G	0.73	0.51	0.69
A	0.43	0.69	1.61
V	0.13	0.07	0.51
L	0.45	0.25	0.56
I	0.39	0.15	0.37
M	1.61	1.19	0.74
S	2.48	1.77	0.72
T	0.57	0.35	0.61
P	1.71	1.13	0.66
N	2.65	1.98	0.75
Q	0.32	0.32	1.00
F	0.87	0.67	0.77
Y	0.61	0.49	0.80
W	0.30	0.39	1.30
K	0.10	0.09	0.88
R	0.76	0.53	0.69
H	1.25	1.11	0.89
D	[3]ND	ND	ND
E	4.05	3.61	0.89

Table 1. Fractional urinary L-amino acid excretion

[1]Fractional excretion was calculated using an estimate of the glomerular filtration rate of 240 ul/min in males (Qi et al, 2004), the measured amino acid concentrations in plasma and urine and the volume of urine excreted over 24 h. [2]Males n=5. [3]ND: not determined.

9.3.2. Lack of ACE2 affects mouse development

To test whether the disturbances in plasma amino acid homeostasis affected mouse development, *ace2* null mice and wild-type littermates were placed in metabolic cages. This allowed measurement of basic parameters such as food and water intake as well as urine and faeces output. Urine and plasma were collected and analysed further (Table 2). Body weight (BW) was slightly but significantly lower in *ace2* null mice, when compared to their wild-type littermates.

Disturbances in ion excretion (higher excretion of Ca^{2+}, Mg^{2+} and PO_4^{3-} and lower excretion of SO_4^{2-} in *ace2* null mice) and pH might be linked to differences in transporters expression at the small intestine membrane or to Ang II levels. However, Na^+ levels which are more directly linked to blood pressure control were found to be normal. It was however not possible to

measure plasmatic ion levels preventing educated guesses regarding the origin of the disturbance.

Water consumption, when related to the body weight was slightly higher in *ace2* null mice when compared to wild-type littermates, which could be due to increased circulating Ang II levels that stimulate thirst and prevent water loss. However, this might be an artefact as urine volume is not significantly affected.

	[1]*ace2*$^{+/y}$	[2]*ace2*$^{-/y}$	
[3]BW (g)	25.87 ± 0.23	24.90 ± 0.33	*
Food (g)	3.80 ± 0.18	3.83 ± 0.14	
Water (g)	5.02 ± 0.33	5.74 ± 0.29	
Urine (g)	1.20 ± 0.12	1.25 ± 0.12	
Feces (g)	1.38 ± 0.10	1.36 ± 0.10	
/ BW (g)			
Food	0.147 ± 0.007	0.154 ± 0.005	
Water	0.194 ± 0.012	0.231 ± 0.012	*
Urine	0.046 ± 0.004	0.050 ± 0.005	
Feces	0.053 ± 0.004	0.054 ± 0.003	
Urinary parameters			
Anions (umol/24h)			
Cl^-	309.6 ± 14.17	304.7 ± 23.65	
PO_4^{3-}	114.9 ± 8.60	146.8 ± 9.81	*
SO_4^{2-}	70.51 ± 4.71	48.19 ± 3.06	***
Cations (umol/24h)			
Na^+	164.6 ± 14.41	181.8 ± 16.96	
K^+	504.9 ± 33.03	522.0 ± 42.12	
Ca^{2+}	2.21 ± 0.45	5.96 ± 0.98	**
Mg^{2+}	39.72 ± 2.14	67.65 ± 5.55	***
Osmolality (mOsm/kg)	3600 ± 422.6	3716 ± 389.6	
pH	6.26 ± 0.05	6.01 ± 0.03	***
Creatinine (umol/24h)	6.31 ± 0.57	4.69 ± 0.53	
Urea (mmol/24h)	2.07 ± 0.17	2.23 ± 0.10	
Plasma parameters			
Urea (mM)	9.01 ± 0.23	10.01 ± 0.33	*

Table 2. Summary of metabolic cages and urine data from 8 weeks old male mice: [1]*ace2*$^{+/y}$ n=10, [2]*ace2*$^{-/y}$ n=11. [3]BW: body weight. Mean ± SEM. * P<0.05, ** P<0.01, *** P<0.001.

The normal food consumption together with the decreased weight of the of ace2 null mice suggests that defect in amino acid transport is compensated for, and does not affect body weight homeostasis in the adult. To test whether this decreased weight already originates at birth or only later during development, *ace2* male pups from 4 *ace2*$^{+/-}$ x *ace2*$^{+/y}$ breeding pairs were weighed from day 1 to day 47. This shows that *ace2* null mice and wild-type littermates have similar weights at birth and that *ace2* null mice thrive but with a delayed weight gain after weaning starting at day 23. The lower weight of *ace2*$^{-/y}$ was maintained until day 47 (Figure 2). The slower weight gain of *ace2* null mice occurs after weaning of the pups, leading to a diet switch from mother milk to pellet food. This might be linked to a later expression of peptide transporters during development though one can not exclude the impact of ACE2 defect as a key enzyme in the RAS.

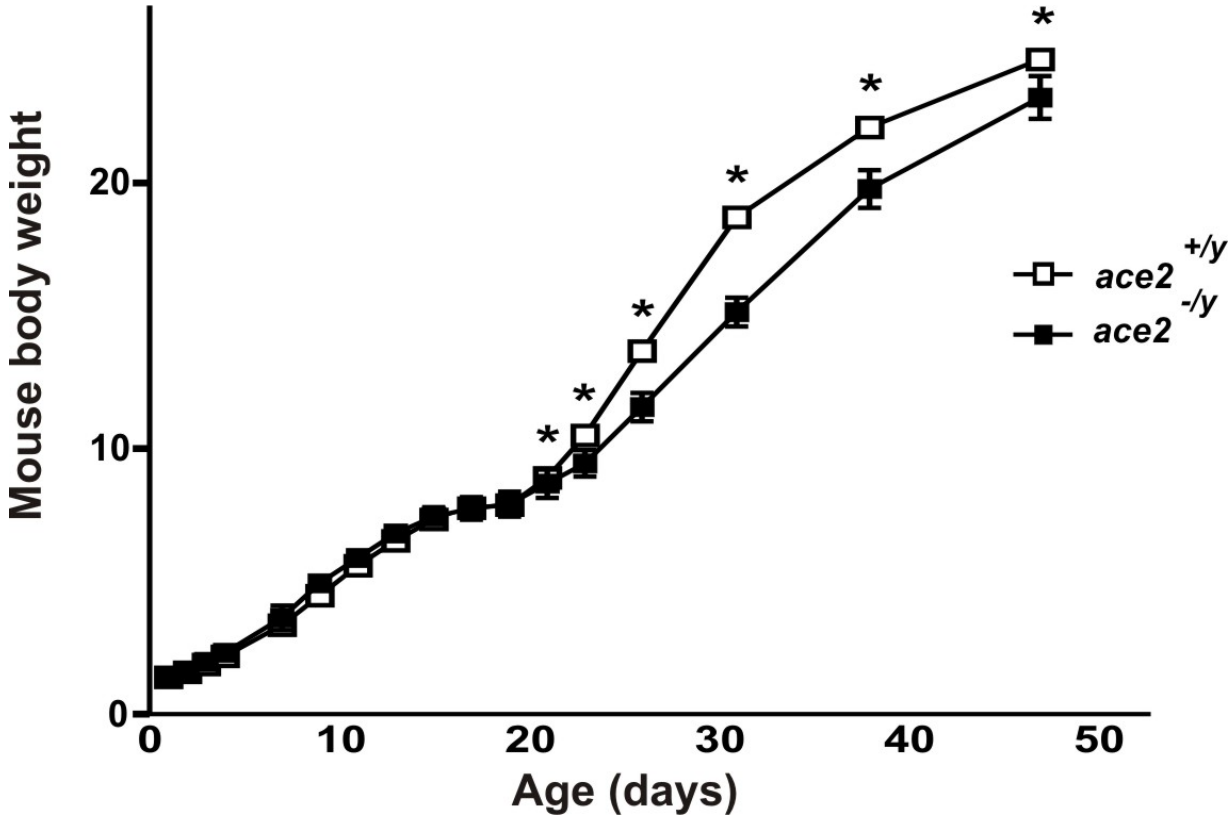


Figure 2. The *ace2* null mice lower weight originates after weaning. The ace2 mice were weighed after birth and showed a slower weight gain after weaning. *ace2*$^{+/y}$ n=8, *ace2*$^{-/y}$ n=6. Mean ± SEM. * P<0.05; ** P<0.01; *** P<0.001. Mouse weight was analysed by paired t-test of the genotype weight average of each litter.

9.4 Discussion

We have determined that the enzyme of the renin-angiotensin system (RAS) ACE2 is critical for the expression of the neutral amino acid transporter B^0AT1 at the luminal membrane of small intestine epithelial cells and, by co-immunoprecipitation, that they interact at the membrane. The extent of this interaction is not clear as we do not know at which point in the sorting process it starts, whether all B^0AT1 molecules still interact with ACE2 once they reach the membrane, and/or whether ACE2 regulates B^0AT1 function at the membrane.
The aim of this last section was to characterise the *ace2* null mice in a broader physiological context. The phenotype of the *ace2* null mice turned out to be relatively discrete when compared to wild-type mice: 1) The plasma levels of L-tryptophan and glycine are decreased and that of L-lysine and urea increased whereas the renal amino acid fractional excretion rates are normal. 2) Additional disturbances in urinary pH and ions were observed. 3) A slower weight gain of *ace2* null mice after weaning led to a lower weight at 8 weeks.
These different points are addressed in the following sections.

Plasma neutral amino acids are compensated by PepT1 absorption

L-isoleucine (L-Ile) is an essential amino acid that cannot be synthesised by the body. As *ace2* null mice do not display Na^+-dependant transport of L-Ile, this amino acid probably enters the enterocytes as di- and tri-peptides through PepT1, thereby compensating plasma L-Ile levels. A way to verify and bypass this compensation would be to replace protein amino acids from the diet with free amino acids and measure the difference between *ace2* null and wild-type mice. Another similar approach would be to inhibit PepT1 with Lys[Z(NO(2))]-Pro, which has only been tested *in vitro* (Knutter et al, 2001) or cross *ace2* null mice to *pepT1* knock-out mice (Hu et al, 2008). The apical exchanger $b^{0,+}AT$-rBAT remains an alternative pathway for L-Ile uptake.

Plasma amino acid homeostasis disturbances observed in *ace2* null mice may, next to small intestine transporter defects, additionally originate from dysfunctions present in other organs at the transporter or at the metabolic level. We have shown that kidney amino acid transport is not grossly modified, but liver, muscle and brain still remain to be investigated.

Plasma L-tryptophan

Though the affinity of B^0AT1 for the essential amino acid L-tryptophan is weaker than for other large neutral amino acids *in vitro* (~25%), the decreased L-tryptophan level in *ace2* null mice is interesting in regards to Hartnup disorder. L-tryptophan indeed serves as a precursor for the synthesis of serotonin, melatonin and niacin (vitamin B_3, nicotinic acid) and its lack might cause Hartnup disorder symptoms that accompany aminoaciduria in certain subjects such as pellagra-like rashes and cerebellar ataxia (Milne, 1969).

Pellagra is a disease characterised by dermatosis, diahrrea, and dementia that manifests in situations of inadequate dietary niacin intake or lack of its precursor L-tryptophan (Pitche, 2005). Niacin is implicated in the synthesis of nicotinamide dinucleotide (NAD) and nicotinamide dinucleotide phosphate (NADP) that are necessary for oxido-reduction reactions, the citric acid cycle and fatty acid synthesis. Niacin defect affects more extensively the brain that has high energy demands, and tissues undergoing high cellular renewing rates like the skin and the intestine. Niacin supplementation has been shown to be effective to treat the ataxia in certain Hartnup disorder cases (Camargo et al, 2008).

The *tmem27* knock-out mice have defective expression of B^0AT1 in kidney but not in small intestine and also do not exhibit modified plasma levels of L-tryptophan. Since the non-aminoaciduria symptoms of Hartnup disorder probably originate from the defect of intestinal B^0AT1 transport, *ace2* knock-out mice might represent an appropriate model. However, Hartnup-related symptoms did not appear in *ace2* null mice possibly due to compensation by PepT1 and because of sufficient niacin levels in the diet (60-70 mg/kg diet). These symptoms could eventually be exacerbated by subjecting the mice to a niacin-free diet.

Plasma Glycine

B^0AT1 has an intermediate affinity for glycine (~50% of L-Leu) (Camargo et al, 2005). Glycine is essential for creatinphosphate synthesis that supplies muscles with energy by replenishing ATP stores. Glycine and L-arginine are metabolised to ornithine and guanidoacetate in the kidney, which is then processed in the liver to creatine and released in the circulation. In tissues and muscle, creatine is phosphorylated to phosphocreatine and serves as high-energy phosphate storage. Hydrolysis of the high energy phosphate bond in muscle leads to conversion to creatinine, which is then eliminated in urine. Measured urinary creatinine levels are normal suggesting that decreased glycine is not sufficient to affect its synthesis.

Urinary creatinine also serves as an indirect measurement of muscle mass, and muscle mostly metabolises branched-chain amino acids, the preferred substrates of B^0AT1. The normal levels of urinary creatinine of *ace2* null mice indicate that muscle metabolism is not affected by loss of intestinal B^0AT1, as expected given the normal plasma levels of branched-chain amino acids.

Plasma L-lysine

The increase of plasma L-lysine is surprising as it is an essential amino acid, ruling out endogenous increases in synthesis. A plasma increase of L-lysine might be due to increased intestinal absorption or rather decreased peripheral tissue uptake or decreased metabolic processing.

Degradation of L-lysine mainly takes place in the liver and mutations in genes involved in L-lysine catabolism cause familial hyperlysinemia (ie. the α-aminoadipic semialdehyde synthase (AASS), the bifunctional protein that contains both lysine-ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH)). Hyperammonemia, though the precise cause is not known, is additionally present in these patients leading to mental retardation and neuromuscular dysfunction (Chen, 2006).
Though *ace2* null mice do not exhibit symptoms caused by hyperammonemia, it is not known yet if there is a decrease of the activity of the enzymes involved in L-lysine catabolism or of L-lysine uptake by the liver.

Another set of mutations leading to disturbances in L-lysine levels are found in amino acid transporter genes $b^{0,+}$AT-rBAT (Slc7a9-Slc3a1) and y^+LAT1-4F2hc (Slc7a7-Slc3a2). Intestinal uptake and renal re-uptake of cationic amino acids in exchange for neutral amino acids is mediated by the heteromeric amino acid transporters $b^{0,+}$AT-rBAT on the apical side and y^+LAT1-4F2hc on the basolateral side.
The defect of the luminal transport leads to cystinuria (OMIM 220100), a condition divided in type A and type B that corresponds to mutations in rBAT and $b^{0,+}$AT, respectively. The only clinical sign of cystinuria is the development of cystine crystals in the urinary tract leading to severe urolithiasis. The knock-out mouse model of *Slc7a9* ($b^{0,+}$AT) displays significantly lower levels of plasma L-lysine and cystine, and only a tendency to display lower L-arginine and ornithine levels. These four amino acids have higher excretion levels in the urine of *Slc7a9* knock-out mice (Feliubadalo et al, 2003). Study of the *Slc3a1* (rBAT) null mice

showed higher levels of L-lysine, L-arginine and ornithine in the urine of these mice and plasma amino acid levels were not measured (Peters et al, 2003).
Similarly, mutations in y^+LAT1 lead to lysinuric protein intolerance (LPI, OMIM 222700). The aetiology of LPI is the impaired (re-)absorption of cationic amino acids in small intestine and kidney, leading to decreased plasma levels and increased urine levels of cationic amino acids. Decreased levels of L-arginine and ornithine that serve as substrates for the urea cycle induce hyperammonemia. LPI is additionally characterised by vomiting, diarrhea, failure to thrive, growth retardation, hepatosplenomegaly, osteoporosis, episodes of coma and chronic renal disease (Sperandeo et al, 2007). Mutations of the associated protein *Slc3a2* (4F2hc) have not been reported in association with LPI which is probably because the knock-out mouse model of *Slc3a2* is embryonic lethal (Tsumura et al, 2003).
Cationic amino acid levels in *ace2* null mice mirror the situation found in the *slc7a9* ($b^{0,+}$AT) knock-out mice such that L-lysine is increased and L-arginine has a tendency to be higher.
A increase in the activity of $b^{0,+}$AT-rBAT is unlikely to rise plasma amino acid levels as dietary amino acids are almost entirely taken up by the small intestine, and $b^{0,+}$AT expression levels at the membrane were not modified in the *ace2* null mice (data not shown). It is however not known how the intracellular concentrations of amino acids are disturbed by the lack of B^0AT1 and how this affects the release of amino acids on the basolateral side, for example by y^+LAT1-4F2hc.

ACE2 association to other transporters

It has been shown for adaptor proteins that they might regulate transporters belonging to unrelated families and/or various functions, as in the case of PDZK1 that regulates PepT1 (Slc15a1) and carnitine/organic cation transporter OCTN2 (Slc22a5) (Sugiura et al, 2008). Next to amino acid transport, unrelated membrane transport proteins like ion channels might be under the control of ACE2 expression, as suggested by their abnormal urinary ion levels. It remains to be seen whether sulphate transporter NaS1 that is expressed in ileum and in proximal tubule cells might be down- or up-regulated, respectively (Morris & Murer, 2001). Decreased urinary phosphate levels on the other hand might be due to increased expression of phosphate transporters NaPi-IIa/c (Slc34a1/3) in the kidney, or decreased NaPi-IIb (Slc34a2) in the ileum (Murer et al, 2004). Similar possibilities can be imagined concerning epithelial calcium channels TRPV5 and TRPV6 in the kidney and Mg^{2+} transporters (Boron & Boulpaep, 2005).
However, a large part of the epithelial transport of ions can be through the paracellular route depending on the diet, possibly relying on tight junction proteins expression such as Claudin

16 for Mg^{2+} transport. Tubular ion reabsorption is also regulated by a variety of other parameters such as the acid-base balance, hormones (parathyroid hormone (PTH), ANP), vitamin D and the levels of other ions. For instance, PTH decreases kidney phosphate reabsorption, but increases calcium and magnesium reabsorption. Therefore it can not be at the origin of the ion disturbances observed in the urine of *ace2* null mice since all these ions are increased (Boron & Boulpaep, 2005).
In the absence of gross phenotype at the level of growth and skeleton, it is likely that the observed changes in urinary excretion are secondary to changes in intestinal absorption.

The ace2 null mice display slower growth after weaning

It is tempting to explain the slower growth of *ace2* null mice by the absence of amino acid transporter B^0AT1. The delay in growth would be secondary to decreased amino acid absorption due to the lack of B^0AT1 that would only be compensated after adaptation to the switch from mother milk to pellet food. It remains unclear why the growth phenotype, if related to nutrient uptake, would only appear after weaning and if the growth defect is generalised or localised to certain organs.
This might be explained by the richness of the mouse milk that contains high amounts of protein, about 10% vs. 0.9% in human milk and 3.3% in cow milk (Boron & Boulpaep, 2005; Hadsell et al, 2007). Similarly, LPI symptoms only appear after weaning due to the relatively low amounts of lysine in breast milk (Cimbalistiene et al, 2007).
As mentioned before, the growth phenotype might also arise due to disturbances linked to the systemic role of ACE2 in the RAS though as yet most of the phenotype associated with increased levels of Ang II has primarily been observed in 6 months old animals.

10. Conclusion and Outlook

The results presented in this thesis demonstrate the importance of tissue-specific associated proteins ACE2 and Tmem27 in intestinal and renal amino acid absorption and reabsorption, respectively.

Our work with the *ace2* and *tmem27* knock-out mice models has shown that correct localisation of B^0AT1 (Slc6a19) to the apical membrane is only possible when tissue-specific associated proteins ACE2 in small intestine enterocytes and homologue Tmem27 in kidney proximal tubule are expressed. Absence of ACE2 accordingly led to the abolition of functional Na^+-dependant L-isoleucine transport. Co-immunoprecipitation of ACE2 with B^0AT1 showed that ACE2 is not only necessary but also directly interacts with B^0AT1 at the luminal membrane of enterocytes.
Thus, *ace*2 and *tmem27* null mice can be used to study the role of the neutral amino acid transporter B^0AT1 in kidney and intestine independently. Further study of *ace2* null mice evidenced a rather discrete phenotype with decreased plasma levels of L-tryptophan and glycine and increased plasma levels of L-lysine and urea. Though renal amino acid fractional excretion rates were normal, *ace2* null mice exhibited disturbances in urinary pH and ions. Finally, the *ace2* null mice grew more slowly after weaning leading to a lower weight at 8 weeks.
Furthermore co-expression of these associated proteins with the human orthologue of B^0AT1 in the heterologous *X. laevis* oocyte system increased the transport rate and the cell surface expression of the transporter. This allowed a more complete characterisation of wild-type and Hartnup-causing mutations of B^0AT1, evidencing different classes of mutants.

B^0AT3 (Slc6a18), that is expressed in kidney late proximal tubule and not in small intestine needs association to Tmem27 for expression *in vivo*, similarly to its homologue B^0AT1. However, only co-expression of ACE2 and not Tmem27 supported the functional expression of B^0AT3 in *X. laevis* oocytes that enabled us to measure its function as a transporter of neutral amino acids. B^0AT3 thereby exhibited a high affinity ($K_{0.5}$ = 0.21 mM for L-Ile) Na^+- and Cl^--dependant transport of a broad range of neutral amino acids.
Re-analysis of the *slc6a18* mouse model after more than 10 generations of back-cross in the C57Bl/6 background confirmed aminoaciduria though strikingly, there was not only a substantial urinary loss of glycine (60-fold higher than in $xt2^{+/+}$) but also of L-glutamine (20-fold) and of all other neutral amino acids. This confirms the complementary role of B^0AT3 to

B^0AT1, reabsorbing the leftover tubular amino acids or the amino acids that backleak through the paracellular route in the late segments of the proximal tubule.
In their original description, *slc6a18* null mice displayed a significantly elevated blood pressure. We could however only observe a stress-sensitive hypertension and a stress-induced increase in respiratory rate in *slc6a18* null mice, suggesting a possible impact of the lack of this transporter on regulatory functions of the sympathetic nervous system or on circulating L-arginine levels thereby affecting NO synthesis.

In conclusion, the association of intestinal B^0AT1 with a key regulator of the RAAS is surprising, and raises many interesting questions regarding the role of ACE2 as a regulator of Ang II levels in the small intestine and the physiological relevance of its association with B^0AT1.

How is RAAS affecting nutrient transport in the small intestine ?

It remains to be investigated whether luminal ACE2 regulates Ang II levels in the lumen of the small intestine and how this may affect amino acid transport and absorption of other substrates as well as enteric blood flow. Additionally, ACE2 peptidase activity on ghrelin might also regulate enteric nutrient transport (Vickers et al, 2002).
Such a broader effect is suggested by the regulation of glucose uptake by Ang II and by the modified glucose homeostasis of *ace2* null mice (Niu et al, 2008). Incubation of increasing concentrations of Ang II decreased glucose uptake levels in intestinal ring segments, an effect that was inhibited by AT1 receptor antagonist losartan (Wong et al, 2007). This implies that if endogenously produced Ang II secreted in the lumen of the small intestine regulates SGLT1 expression, *ace2* null mice will display higher concentrations of Ang II and therefore less glucose uptake. To further test this hypothesis, we will measure glucose uptake in intestine rings of *ace2* null mice. Similarly, additional treatment of the mice with losartan and ACE inhibitors will show if Ang II levels affect glucose or amino acid uptake and if this is dependant on AT1 receptor.

Is ACE2 association to B^0AT1 relevant ?

It is hypothesised that ACE2 arose from an ancient chimeric gene duplication of ACE and Tmem27, which are both already present in the pufferfish (Chou et al, 2006; Mount, 2007). It is however not clear why ACE2 does not play a role on amino acid transport in kidney, and why ACE2 replaced the role of Tmem27 in small intestine. It could be relevant for an amino acid transporter to be associated to an exopeptidase, however the specificity of ACE2 for *Pro-(1-3 residues)-Pro-hydrophobic amino acid* patterns might be too high for meaningful substrate channelling. It is to be investigated whether other brush border peptidases or disaccharidases may, like ACE2, associate to other amino acid transporters or glucose transporters, respectively.

Does ACE2 associate with other members of the SLC6 family ?

It remains to be examined whether ACE2, like Tmem27, interacts with other amino acid transporters of the SLC6 family. The Na^+ and Cl^--dependant neutral and cationic amino acid transporter $ATB^{0,+}$ (Slc6a14) is hypothesised to be expressed in rabbit ileum (Anderson et al, 2008) and in human colon (Gupta et al, 2005). Considering that the Na^+-dependant transport of L-isoleucine is absent in all segments of the small intestine of the *ace2* null mice (data not shown), $ATB^{0,+}$ might either be downregulated, or not expressed in mouse small intestine.
Preliminary results show that luminal expression of the imino acid transporter SIT1 (Slc6a20) is decreased in the small intestine of *ace2* null mice as seen by immunofluorescence. Though this interaction remains to be further characterised, testing of SIT1 functional expression by uptake of L-Proline in everted intestinal rings is promising. Additionally, results obtained by R. Vuille and Dr. S.M.R. Camargo by co-expressing human SIT1 with ACE2 in *X. laevis* oocytes suggest functional interaction of the two proteins.
Given the expression of SIT1 in brain and lung, further focus will be aimed at its possible interaction with associated proteins ACE2 or Tmem27 in those tissues.

The ace2 null mice – A model for Hartnup disorder ?

Mutations in B^0AT1 are the only known cause of Hartnup disorder, a condition which is characterised by neutral aminoaciduria and various other clinical symptoms such as pellagra-like rash, cerebellar ataxia and other neurological dysfunctions that appear to mostly manifest in situations of protein-restricted diet. Though *tmem27* null mice suffer from extreme

aminoaciduria due to the lack of renal B^0AT1 and other transporters, plasma amino acid levels are not affected (Danilczyk et al, 2006). Additional Hartnup symptoms are therefore probably not caused by the renal defect in itself, and could be better studied with the *ace2* null mice.

Indeed the defect of intestinal B^0AT1 might suffice to lead to decreased plasma levels of L-tryptophan, the precursor of niacin and serotonin, leading to neurological and dermal symptoms. It remains to be investigated how lower levels of circulating L-tryptophan affect tissue concentrations of this amino acid, and if this is sufficient to induce neurological and pellagra-like symptoms when combined with low-protein diets.

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12. Curriculum Vitae

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Education

08/2005 - 03/2009	Employed as a PhD student at the University of Zurich since August 2005. Institute of Physiology, University of Zürich, Switzerland, in the group of Prof. F. Verrey
03 - 06/2005	Work as a biologist in the Département de Pharmacologie et Toxicologie, Lausanne (VD), Switzerland
1999 - 2005	Biologist Diploma at the University of Lausanne (VD) in physiology : Study of the potential function of RGS2 in the kidney. Dr. D. Firsov group ; Institute of Pharmacology and Toxicology, Lausanne
1996 - 1999	Baccalauréat Vaudois ès Sciences in Gymnase de Chamblandes (Pully, VD), Maturité Fédérale de type mathématiques-sciences
1991 - 1996	Ecole secondaire in Vevey : Certificat d'Etudes Secondaires, division prégymnasiale, section mathématiques-sciences

Fellowships, Prizes

2006-2008	2 years fellowship from the University Research Priority Program "Integrative Human Physiology at the University of Zurich.
2007	Poster Prize at the 3rd ZIHP Symposium

Publications

Singer, D.*, Camargo, S. M.*, Huggel, K., Romeo, E., Danilczyk, U., Kuba, K., Chesnov, S., Caron, M.G., Penninger, J. M., Verrey, F. Orphan transporter SLC6A18 is renal neutral amino acid transporter B^0AT3 *J Biol Chem*. In press. * *Shared first author*

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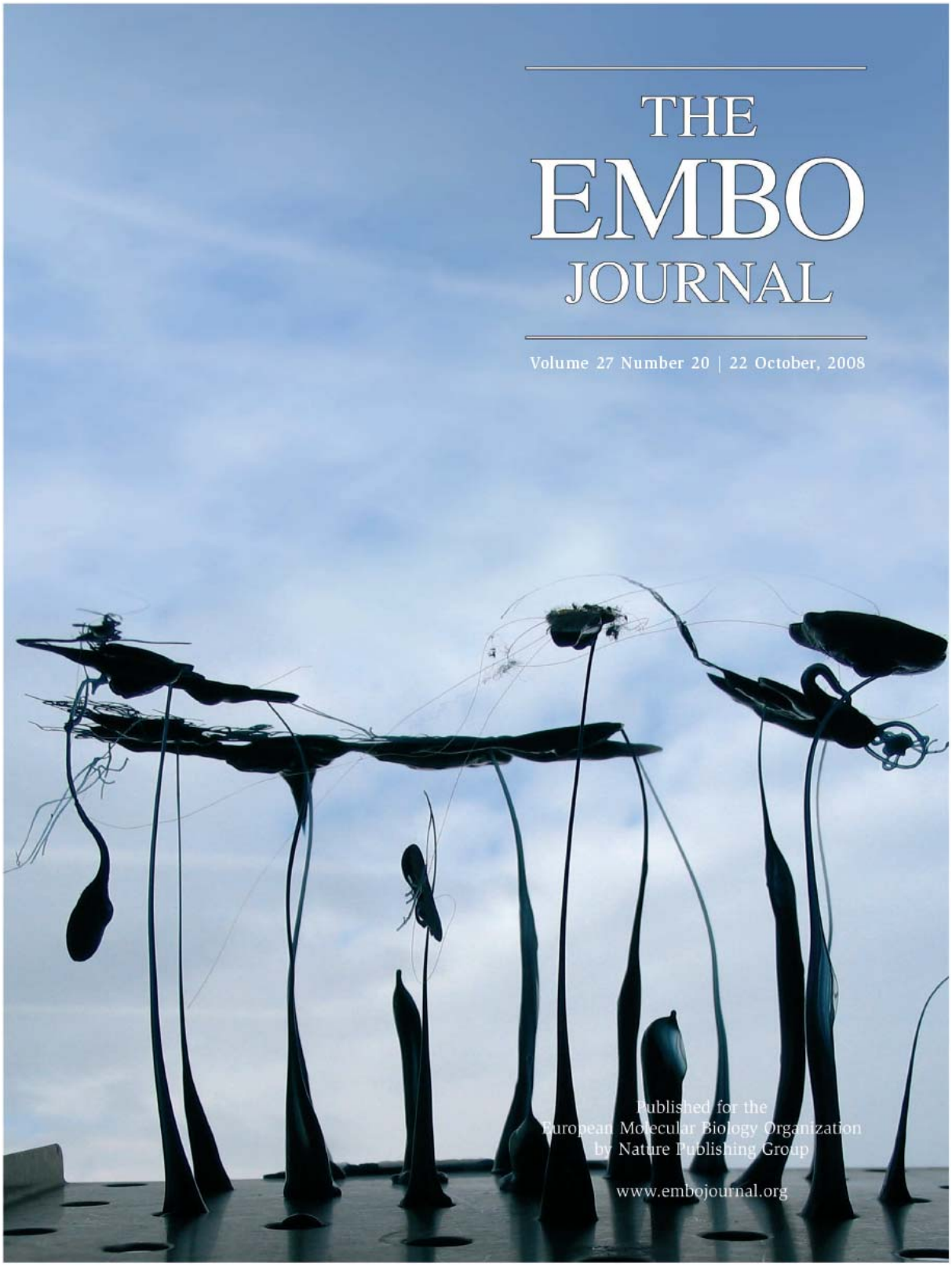
The EMBO Journal Cover, 2008 Oct 22, 27(20):2639-2808 depicting a box of pipette tips that has melted through the grid of a (unfortunately too hot) glassware dryer. The photographer, Dustin Singer, is a PhD student at the Institute of Physiology of the University of Zurich, Switzerland, and has a keen interest in photography and juggling.



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